FRACTIONATION OF HEAVY METALS

IN NATURAL SAMPLES

by

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MEMORANDUM

The research described in this dissertation was carried out in the Department of Inorganic Chemistry at the University of Bristol, under the supervision of Dr. G. Nickless, between October 1981 and April 1984. It was the independent work of the author and has not been used in any other dissertation, except where otherwise stated.

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ABSTRACT

The thesis consists of eight experimental sections entitled : Freshwater; Metal Speciation, Sediments; Metal Speciation and Fractionation; Soils; Metal Fractionation, Humic and Fulvic Acids; Interactions with Heavy Metals, Plants; Metal Fractionation Studies, Characterisation of Plant Metallothionein-like Protein, Statistics and Investigation of Sources of Contamination.

The introduction is a survey highlighting and emphasising the influence of heavy metals in the environment, their toxicity, associations with clays, organic matter and hydrous oxides, as well as the importance of metal speciation. The direction in which the work proceeded is outlined.

Because of the extreme sensitivity involved in the fractionation studies (FAAS, HMDE and RDE were the analytical methods used), a detailed investigation was carried out into the purification of the reagents employed in the chemical procedures.

Heavy metal speciation in waters and sediments was based upon size fractionation and leaching with extractants of decreasing pH. Membrane filters were used in sequence to separate the metal species into different groupings.

Model compounds (prepared from metal loaded peat and ferric hydroxide) together with SRM 718 Orchard Leaves, were used to evaluate chemical extraction schemes. The sequential leaching schemes differentiate the metal format present in soils and plants, and hence indicated the bioavailability of Cd, Cu, Pb and Zn. Cadmium was often determined in the exchangeable phase, zinc in most fractions, while lead and copper - were generally present in less available forms. The organic-metal associations identified by application of the fractionation schemes were

further investigated by a study of the effect of humic and fulvic acid (chemically extracted from Levington's Universal Mixture) on Cd, Cu, Pb and Zn ions. The results obtained were subject to a number of practical difficulties.

Application of sequential leaching to metal-tolerant plants demonstrated metal-plant component associations - Cd was mainly associated with protein, Cu with protein and low molecular weight materials, Pb with the cell walls and Zn with low molecular weight materials and proteins. The cell walls were important for all the metals studied. The effect of one metal on another was also examined - a high Zn concentration caused increased Cu uptake in all three populations and high Pb concentrations lead to high Cu and Zn concentrations in Merlin and Parys plants, but decreased Cu and Zn concentrations in the Goginan population.

The protein-metal associations elucidated for such plants suggested the possibility of a metallothionein-type tolerance mechanism. The isolation and characterisation of the metal binding material pointed to a peptide rather than the type of protein found in mammals.

The analytical precision and reproducibility of the HMDE and RDE techniques were determined. The conventional limits to the individual parameters of a regression line ($y = f(x)$) assumed that the abscissa variable (x) is controlled and that the variability can be attributed solely to the ordinate variable (y). Since this is inappropriate to a calibration problem Working-Hotelling 95% confidence regions for the entire line have been used. This then combined with tolerance limits for the observations to give the 95% confidence interval for 90% of future observations.

Potential sources of contamination were also identified by means of GFAAS.

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LIST OF ABBREVIATIONS

AAS	atomic absorption spectrophotometry
ac	alternating current
AD	acid digest
AES	atomic emission spectrophotometry
ASV	anodic stripping voltammetry
BDH	British Drug Houses
conc	concentrated
concn.	concentration
dc	direct current
dc ASV	direct current anodic stripping voltammetry
DCP	current-sampled dc polarography
DDW	double distilled water
DEAE	diethylaminoethyl cellulose
DI	deionised water
DM	dry matter
DME	dropping mercury electrode
DPASV	differential pulse anodic stripping voltammetry
DPP	differential pulse polarography
DW	dry weight
$E_{\frac{1}{2}}$	half-wave potential
EDTA	ethylene diamine tetra-acetic acid
E_h	redox potential
ETAAS	electrothermal atomic absorption spectrophotometry
Exch.	exchangeable
FA	fulvic acid
FAAS	flame atomic absorption spectrophotometry
GCE	glassy carbon electrode

GFAAS	graphite furnace atomic absorption spectrophotometry
HA	humic acid
HMDE	hanging mercury drop electrode
HSAB	hard/soft acid base
ICP	inductively coupled plasma
ICPES	inductively coupled plasma emission spectrophotometry
ICP/MS	inductively coupled plasma/mass spectrometry
i.d.	internal diameter
i_d	diffusion curve
ILL	International Laboratories Limited
Insol.	insoluble
IR	infra red
ISE	ion selective electrode
L	length
M(r)	molecular weight
mp	melting point
Mass Spec	mass spectrometry
NAA	neutron activation analysis
NBS	National Bureau of Standards
N.D.	not detected
NPP	normal pulse polarography
PARC	Princeton Applied Research Corporation
PCBs	polychlorinated biphenyls
pH	activity of hydrogen ion ($- \log_{10} H^+$)
Prep	preparative
RCF	relative centrifugal force
RDE	rotating disk electrode
RSD	relative standard deviation
RT	room temperature

SCE	standard calomel electrode
SD	standard deviation
SE	standard error
sol.	soluble
SRM	standard reference material
TEL	tetraethyl lead
Temp.	temperature
TFME	thin film mercury electrode
Thk	thick
Tris	Tris (hydroxymethyl) methalamine
UV	ultra violet
v/v	volume to volume (ratio)
vs.	versus
Wd	width
w/v	weight to volume (ratio)
X	element
Zp	the normal deviate

Units

km	kilometre	MHz	megahertz
cm	centimetre	M	molar concentration
mm	millimetre	mM	millimolar
µm	micrometre	µM	micromolar
nm	nanometre	^{o}C	degrees Celsius
l	litre	^{o}K	degrees kelvin
ml	millilitre	%	percentage
µl	microlitre	ppm	parts per million
g	gram	rpm	revolutions per minute
mg	milligram	meq/g	milliequivalents/gram
µg	microgram	mV/s	millivolts/second
ng	nanogram	nm/s	nanometres/second
pg	picogram	cm/s	centimetres/second
h	hour	mg/s	milligrams/second
min	minute	min/m^3	minutes/cubic metre
s	second	Tg/yr	Teragrams/year
ms	millisecond	mg/kg	milligrams/kilogram
µs	microsecond	kg/yr	kilograms/year
kV	kilovolt	mg/l	milligrams/litre
V	volt	µg/l	micrograms/litre
mV	millivolt	ng/l	nanograms/litre
kW	kilowatt	g/l	grams/litre
mA	milliamp	µg/ml	micrograms/millilitre
µA	microamp	ng/ml	nanograms/millilitre
ml/l	millilitre/litre	mM/l	millimolar/litre
µg/g	microgram/gram	ml/g	millilitre/gram

$\mu A/cm^2/\mu g$ microamp/square centimetre/microgram

N.D. =. Not Detected, i.e. less than :-

FAAS (µg/ml)			
Zn	0.36		0.05
Cd	0.1	and for Tris-HCl	0.05
Pb	0.1		0.05
Cu	0.1		0.01

HMDE (ng/ml)	
Zn	1.0
Cd	1.0
Pb	1.0
Cu	2.0

RDE (ng/ml)	
Cd	0.75
Pb	0.39
Cu	0.69

GFAAS (ng/ml)	
Cd	0.8
Pb	0.5
Cu	0.5

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PART I : INTRODUCTION

SECTION I

1) Heavy Metals in the Environment

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   b) Cadmium

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2) Toxicity of Heavy Metals and the Importance of Speciation

3) Associations and Transport of Heavy Metals

   a) Clays

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4) Heavy Metals in Freshwater and Sediments

5) Heavy Metals in Soils

6) Heavy Metals in Plants

   a) Exclusion/restricted transport

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SECTION I

(1) Heavy Metals in the Environment

During the last twenty years there has been increasing interest in environmental contamination by heavy metals such as cadmium, copper, lead and zinc. Heavy metals can have a profound effect on man and other organisms, because some are essential for the maintenance of normal growth and development, while others are toxic (1-3).

Unlike pollutants such as the polychlorinated biphenyls (PCBs), and organochlorine pesticides, the presence of heavy metals in the environment is a natural phenomenon (3). Heavy metals also pose a special threat in that they are immutable, i.e. not biodegradable in the biogeochemical cycle (4), and there is much concern that they can enter the food chain via plant uptake or contamination of ground water supplies by leaching through the soil (5). Recently, there has been recognition of a significant association between excessive heavy metal accumulation in man and the incidence of diseases (6).

All metallic elements come ultimately from the earth's crust (7), thus the major natural sources of heavy metals are soils, rocks and ores. Among the most important natural sources of heavy metals in the atmosphere are from :-

1. Surface waters - spray evaporates leaving salt particles.
2. Soil particles - continental dusts; some of these may be volcanic; soil movement by wind is prominent in arid areas and on coastlines.
3. Vegetation - organic particles evaporated from forest vegetation, together with pollen and spores.
4. Soot particles - forest, grass and bush fires (8).

Table 1 gives an estimate of the relative importance of the natural and anthropogenic sources of particulate matter in the atmosphere (9).

Table 1 : Source strengths for primary atmospheric particulate matter (Teragrams (10^{12}g)/year)

Source	Strength (Tg/yr) Natural	Anthropogenic
Primary particle production :		
Fly ash from coal	-	36
Iron and steel industry emissions	-	9
Non-fossil fuels (wood, mill wastes)	-	8
Petroleum combustion	-	2
Incineration	-	4
Agricultural emission	-	10
Cement manufacture	-	7
Miscellaneous	-	16
Sea salt	1000	-
Soil dust	200	-
Volcanic particles	4	-
Forest fires	3	-
	1207	92

Table 2 indicates the level of cadmium, copper, lead and zinc in a 1974 London dust sample (8).

Table 2 : Levels of Cd, Cu, Pb and Zn in a London air sample (1974) in mg X/kg dust

X	mg X/kg
Cd	1 - 47
Cu	8 - 1100
Pb	30 - 7700
Zn	9 - 2500

In surface waters, metals originate to a large extent from :-

1. Bed-rock and surface run-off - the rock and soil of the drainage basin is the most important natural source of metals to the water bodies present in the basin. The amount present varies greatly with rock type and mineral content (9,10). Table 3 shows the average concentrations of cadmium, copper, lead and zinc in some igneous and minor sedimentary rocks (8).

Table 3 : Average concentrations of Cd, Cu, Pb, Zn in a) some igneous rocks (mg X/kg)

	Basalt	Granite	crust
Cd	0.13	0.09	0.11
Cu	90	13	50
Pb	3	24	14
Zn	100	52	75

b) some minor sedimentary rocks (mg X/kg)

	Manganese nodules	Phosphorites	Petroleum	Lignite	Coal
Cd	8	0.01 - 25	0.03	-	0.2
Cu	2600	100	1.3	6 - 25	15
Pb	8700	2 - 14	-	-	10
Zn	710	300	30	30 - 300	50

2. Precipitation or dry fallout on the water surface or in the drainage basin is the second most important source of metals to water bodies (10). An estimate of the atmospheric input to the ocean, of cadmium, copper, lead and zinc, is given in Table 4.(8).

Table 4 : Estimates of atmospheric input to ocean in kg/yr (8)

X	Input/kg yr^{-1}
Cd	10^8
Cu	2×10^8; 3.6×10^7
Pb	3×10^8
Zn*	107*

* Concentration of zinc in rainwater only (µg/l) (10).

Man's activities have increased the quantity and distribution of heavy metals in the atmosphere, on the land, and in rivers, lakes and seas (11). The major anthropogenic sources of metals in the environment are :-

i) Combustion of fossil fuels.

ii) Mining and smelting operations.

iii) Processing and manufacturing industries.

iv) Waste disposal, including dumping, release of domestic sewage and scrap metal handling.

v) Farming and forestry - through the use of fertilizers and pesticides and because both increase soil erosion (12).

The exposure of the majority of the human population to metal pollutants present in the environment appears to be predominantly via ingestion. Inhalation makes a relatively minor contribution to man's

body burden, with the possible exceptions of people living close to a significant emission source (13) or people exposed to metal contamination via their occupation (14).

The increasing exposure of biological systems to heavy metals has stimulated investigations into the extent of contamination and sources of entry of heavy metals into the environment (2,6).

It is now realised that knowledge of the total metal concentration alone is not always sufficient, but in order to understand thoroughly the distribution and ecological implications of trace metals, the physico-chemical form (i.e. speciation) of the metal must be elucidated (15). From a public health viewpoint it is important whether the trace metals are :-

i) in solution or adsorbed on solids (where they are readily available),

ii) in organic materials or precipitated hydrous manganese and iron oxides. On such a surface chemical changes e.g. oxidation of organic matter or reduction of iron and manganese hydroxides are required before heavy metals are released, so that they are rated as less available, or

iii) in crystal structures of suspended materials where they are almost unavailable in nature (16).

Thus the ecological significance of heavy metal pollution depends not only on their relative abundance but on their speciation (17). Variation in the speciation of trace elements can dramatically change their bioavailability or toxicity (18). The significance or hazard associated with the accumulation of such elements in soils depends on their potential for plant uptake and for leaching into ground water (19). For example, at Shipham, Somerset, the cadmium content of potatoes from contaminated soil was 0.56 mg/kg dry matter (20). Spinach grown on two

soils of similar pH, but different organic content, was found to contain 4.5 mg Cd/kg dry matter from the soil of high organic content and 5.5 mg Cd/kg from the soil of lower organic content (19). Acid mine drainage led to contamination of the River Jintsu, Japan, which in turn led to the chronic condition of cadmium poisoning known as Itai-Itai disease (21). A number of major studies have indicated that heavy metals are accumulated by plants to high concentrations (6, 22-27).

Although some metals such as zinc and copper are essential to organisms for the maintenance of normal growth and development (3), others such as cadmium and lead (which are present ubiquitously) are not known to be essential. Both essential metals and those thought to be non-essential can accumulate in biological systems and all may reach toxic levels in various food chains (8,28).

It is best to discuss zinc, cadmium, lead and copper individually, since the majority of studies have dealt with only single metal effects (29).

a) Zinc

Zinc occurs in several mineral forms (29), being found as the sulphide, carbonate, and hydrated silicate ores, frequently accompanied by other metals, mainly iron and cadmium (30). The principal zinc ores are sphalerite (ZnS), wurtzite (ZnS, hexagonal) (12), and calamine ($ZnCO_3$) (29). Zinc concentrations in soils range between 60 and 2000 mg/kg (8).

In that zinc has a full 3 d-shell, Zn is not a transition element, but does have the transition element properties of forming complexes and being a strong Lewis acid. In aqueous solution there is no evidence of Zn (I) or of oxidation states higher than II. Zinc (II) has the ability to occupy low symmetry sites in enzymes (30) and is an essential constituent of several enzymes present in all phyla. Zinc is a component

of superoxide dismutase, carboxypeptidase, carbonic anhydrase, and a range of dehydrogenase enzymes (31). These enzymes participate in a wide variety of metabolic processes (32).

The mine and smelter production of zinc for the years 1950, 1960, 1970 and 1977 are shown in Table 5 (33).

Table 5 : a) Mine production of zinc (content) by continent, 1950, 1960, 1970 and 1977 (thousand metric tons)

CONTINENT	1950	1960	1970	1977[a]
North America	1074	1051	2010	2019
South America	121	219	399	634
Europe	553	1131	1684	2000
Africa	145	260	251	261
Asia	56	355	631	763
Oceania	201	322	489	491
Total	2150	3338	5464	6168

b) World smelter production of slab zinc by continent 1950, 1960, 1970 and 1977 (thousand metric tons)

CONTINENT	1950	1960	1970	1977[a]
North America	1004	1015	1295	1073
South America	9	48	106	151
Europe	798	1458	2140	2820
Africa	23	84	134	191
Asia	49	298	892	1109
Oceania	85	122	260	257
Total	1968	3025	4827	5601

[a] estimate

The world consumption of zinc for 1977 was 5692.7 thousand metric tons.

Galvanising accounts for almost 40% of slab zinc consumption in the United States and 25% in the United Kingdom (33). Zinc is also used in the manufacture of brass and other alloys (12), in some lubricating oils, in tyres (34), paints, batteries and pesticides (12). Other anthropogenic sources of zinc are fertilizers and sewage sludge, the zinc concentration in the former ranges from 17 to 3070 mg kg^{-1} (35) and in the latter approximately 0.65% of the dry matter is zinc (36). Combustion of zinc containing fuels leads to annual transfers of 22 x 10^6 kg zinc/year to the atmosphere compared with 4040 x 10^6 kg zinc/year from mining (8). Buchauer found zinc levels of between 50,000 and 80,000 mg kg^{-1} in the upper soil horizon near zinc smelters in Palmerton, USA (37), while Roberts and Johnson found 11,000 mg zinc/kg soil close to waste materials at a lead-zinc mine complex at Minera, Wrexham (17).

Zinc is readily taken up by plants and is relatively mobile within the plant (38). It is essential to multiple processes including DNA and RNA stability (39). However, even essential elements can become harmful when in excess. For zinc the phytotoxic content is over 400 mg/kg for a number of plants (40). The adverse effects of zinc in many plant species is associated with the inhibition of root growth and chlorosis (31). The adverse effects on the growth of oats, clover and radishes begin to occur at levels of acetic-acid extractable zinc in excess of 200 mg/kg in the soil. Lettuces appear to be considerably more susceptible, inhibition of growth appears to commence around a level of 60 mg/kg 'available' zinc (41).

In man the following types of toxic reactions to zinc have been reported (42) :-

1) 'Metal fume fever' - characterised by pulmonary manifestations,

fever, chills and gastroenteritis has been reported to occur in industrial workers exposed to fumes (42).

2) Ingestion - symptoms include vomiting, lethargy and muscular incoordination. Gastrointestinal bleeding has been observed after ingestion of 220 mg of zinc sulphate, twice daily. Such symptoms were also observed after 12 g of zinc sulphate was ingested over a period of 2 days. Death is reported to have occurred after ingestion of 45 g of zinc sulphate (the normal daily human requirement is between 15 to 20 mg/day) (42).

(b) Cadmium

Cadmium is a strongly chalcophillic element, i.e. it is concentrated in sulphide minerals, a characteristic in which it follows zinc and mercury, and to a lesser extent lead and copper. Few cadmium minerals are known : greenockite (hexagonal CdS), hawleyite (cubic CdS), cadmoselite (CdSe), monteponite (CdO), otavite ($CdCO_3$) and saukovite or cadmium metacinnabar [(Hg,Cd)S]. All are rare and not used for commercial sources (43). Characteristically, cadmium occurs as a contaminant of other sulphide minerals especially zinc sulphides which contain 0.2 - 0.4 per cent cadmium (44). Concentrations as high as 5% have been reported. Fourteen other metal sulphides have also been reported to contain over 500 mg Cd/kg, the most important of these include galena (PbS), metacinnabar (HgS) and chalcopyrite ($CuFeS_2$) (43). Cadmium concentrations in most soils range between 0.5 to 1.0 mg kg^{-1}, but concentrations of over 20 mg/kg occur naturally in some places in England (44). Cadmium is a relatively rare element, the mean crust concentration is shown in Table 6 in comparison with the concentrations of copper, lead and zinc.

Table 6 : Mean crust concentration in mg X/kg of cadmium, copper, lead and zinc

	Mean crust mg X/kg
Cd	0.11
Cu	50
Pb	14
Zn	75

Cadmium is present in a wide range of animal and plant species, but like lead, a biological function has not been defined (45).

Primary cadmium is recovered entirely as a by-product from residues obtained during the smelting of other metals especially lead and zinc. Once used, cadmium, unlike lead for example, is very rarely recovered. For this reason cadmium is often referred to as the "dissipated" element (45).

World production of cadmium in the early 1970s was around 17,000 tons annually, major economic sources are Mexico, Australia, Belgium, Luxembourg, Canada and Peru (46). Cadmium is used in photography, pesticides, nuclear reactors, pigments, plastics stabilizers, alloys and batteries (12). Table 7 lists the uses of cadmium in the US along with the relative dissipation and emissions during 1968-1972 (47).

Table 7 : Summary of US Cadmium flow, dissipation and emissions during 1968-1972. Quantities in 10^3 kg/year (47)

Source	Commercial Flow	Dissipations in End Products	Airborne Emissions	Waterborne Effluents	Land-Destined Wastes
In domestic zinc ores	2,250				
Losses in Beneficiation			0.2		250
In domestic zinc concentrates	2,000				
In imported concentrates	600				
Total to zinc Smelters	2,600				
Losses in zinc Smelting			102	7	
In zinc for galvanizing		160			
Corrosion in galvanized products					40
Losses in scrap processing			0.4		12
In ZnO for rubber		15			
Rubber tire wear			5.2		
Net from zinc smelting	2,300				
In domestic flue dusts	700				
In imported flue dusts	400				
Domestic cadmium metal production	3,400				
Cadmium metal from GSA stockpile	500				
Cadmium metal imports	1,700				
Total cadmium metal supply	5,600				
Cadmium metal to electroplaters	3,100				
losses in electroplating				7	77
In electroplated products		3000			
Losses in scrap processing			10		318
Cadmium metal to pigments	700				
Losses in processing			9.5	0.8	16.5
In plastics		675			
Losses in incineration			6		26
Cadmium metal to batteries	230				
Losses in processing			0.7	0.3	9
In batteries		220			
Cadmium metal to alloys and other uses	390				
Losses in processing			2.3		
In alloys, etc.		390			
Losses in scrap processing			2.2		20
From phosphate fertilizers					100
From phosphate detergents				10	
Collected in sewage sludge			20		250
From coal combustion			80		370
From oil combustion			50		
From lubricating oils			0.8		
Grand totals	27,640	5630	302	25	1532

Other sources of cadmium include phosphatic fertilizers (1.2 to 79 mg Cd/kg)(35), lubricating oils, motor vehicle tyres, coal and oil combustion (average Cd content of coal is 1 mg/kg and for oil 0.5 mg/kg), refuse incineration and sewage sludge (concentration ranges from 3 to 79 mg Cd/kg dry solids (13)). Table 8 indicates the end uses of cadmium in the USA, UK and Japan for 1965, 1970 and 1974 (43).

Table 8 : End uses of cadmium in the USA, UK and Japan. Quantities are in metric tons (43)

	USA			UK			Japan		
End Use	1965	1970	1974	1965	1970	1974	1965	1970	1974
Batteries	279	136	544	117	110[a]	180[a]	51	176	320
Pigments	1160	587	997	272	335	416	185	444	252
Stablizers	385	1081	906	136	168	207	161	341	173
Plating	2447	2052	2718	493	490	457	168	135	5
Alloys	b	b	b	178	133	96	42	169	99
Others	408	251	441	74	77	85	180	220	78
Total	4679	4107	5605	1270	1313	1441	787	1485	927

[a] Estimated
[b] Included in "Others"

The transfer of cadmium to the atmosphere due to mining is 7.7×10^6 kg/yr and that by combustion 0.065×10^6 kg/yr (8). Buchauer found from 900 to 1500 mg/kg of cadmium in the upper soil horizon in the vicinity of two zinc smelters in Palmerton, USA (37).

Cadmium is readily absorbed, translocated and accumulated in various organs of crop species at relatively low concentrations of the metal within the soil root zone (28). At low concentrations Cd^{2+} is not toxic to plants, but at concentrations greater than 100 µg/kg Cd^{2+} characteristically causes leaf chlorosis, accompanied by a lowering of photosynthetic rate (31). The rapid uptake and translocation of the metal is of concern as cadmium has a tendency to be accumulated by mammals in the liver and kidneys (44). In man, kidney damage, which is the earliest symptom of chronic cadmium intoxication, may occur in sensitive individuals whose daily intake exceeds 200 µg. The level for most of the population is a daily intake of cadmium in the range 350 to 600 µg sustained for 50 years. The threshold concentration for toxicity is 200 mg/kg wet weight in the kidney cortex, which is roughly equivalent to 30 mg/kg in the liver and 10-15 mg Cd/kg creatinine in the urine. With acute ingestion, the symptoms are severe nausea, vomiting, diarrhoea and muscular cramp. For humans the no-effect threshold of a single oral dose of cadmium as a salt is about 3 mg. The human inhalation lethal dose is slightly less than 3000 mg Cd min/m^3 - symptoms do not develop immediately, but death can occur following pulmonary oedema (44). Softening of the bone tissue as found in Itai-Itai disease is thought to occur after a daily intake of 600 µg cadmium (48). In the ionic form, the element can interact with polynucleic acids (DNA, RNA) and with some phospholipids. The binding of cadmium to heterocyclic bases can result in the breaking of hydrogen bonds and destabilisation of the DNA structure (45). Similar to zinc, cadmium has a completed 4 d-shell of electrons and shows some of the similar chemical behaviour to zinc, although cadmium cations are considered to be softer acids compared to zinc. Hence, Cd^{2+} may substitute Zn^{2+} in many zinc enzymes, so altering their activity (7,45).

(c) Lead

Lead is the most abundant of the natural heavy elements, with atomic numbers greater than 60. The primary form of lead in the natural state is galena (PbS), but is also found as plattnerite (PbO_2), cerussite ($PbCO_3$) and anglesite ($PbSO_4$) (49). Galena occurs mostly in deposits which also contain zinc minerals and small amounts of copper, iron and a variety of trace elements (12). The range of lead concentration in arable soils is from 0.8 to 500 mg/kg, but levels up to 45 mg/g have been reported (49).

Lead is a minor natural constituent of soils (ranging from 2 to 300 mg/kg) and of plants (ranging from 1 to 13 mg/kg) (8) but has no known biological function (45).

The principal anthropogenic sources of lead are combustion of lead containing fuels used in internal combustion engines (the lead is chiefly exhausted from automobiles as lead halides and ammonium lead halides (50)), combustion of lead containing coal or fuel oil, and lead mining and refining (51). Lead is also used in storage batteries, pigments, ammunition, solders, cable covering, anti-fouling paints and alloys (12) and has been used in pesticides (52). Sewage sludge is another potential source of lead (13). Table 9 summarises the lead consumption in Europe, the USA and Japan (the major lead consumers in the Western world) (53).

Table 9 : Lead consumption by use in Europe, USA and Japan (x 1000 metric tons)

	1974	1980 (estimated)
Batteries	1390	1700
Pigments & chemicals	360	500
TEL	317	200
Alloys	288	300
Cable	322	200
Pipe & sheet	173	200
Miscellaneous	258	200
Total	3108	3300

The total western world consumption for 1980 was estimated at 4100 x 1000 metric tons (46). The annual transfer of lead to the atmosphere from mining is estimated to be 3340 x 10^6 kg/yr, while from combustion of lead containing fuels (mainly petrol) this value is estimated as 183 x 10^6 kg/yr (8).

Lead, like copper (31) and unlike zinc (38) and cadmium (28) is not readily taken up from soils by plants (54-56). Actual uptake and association of lead with plants is affected (to varying degrees) by almost all environmental factors (57). A stunting effect on corn (Zea mays) has been demonstrated from as little as 24 µg lead per gramme of sand under phosphate-deficient conditions (58). The most confusing aspect of lead toxicology is the lack of any consistent relationship between amounts of lead in soil and the extent of the toxicity. Lead gives rise to a range of toxicity symptoms in plants including (31) :

1. inhibition of root elongation
2. roots may form adventitiously on the stems

3. stem elongation and leaf expansion are inhibited

4. chlorosis and a purple discolouration of foliage.

Some toxic effects in man are given in Table 10 together with the blood lead values at which the effects were noticed.

Table 10 : Blood lead concentrations suggested as the no-response (i.e. not inducing a specific intensity of a specific parameter in 5% of the subjects) or low-response levels for the parameters shown (ug/100 ml) (59)

Parameter	Children	Adults
Anaemia	40 - 50	70 - 80
Encephalopathy	50 - 60	>> 80
Minimal brain dysfunction	>40 - 50	
Peripheral neuropathy	> 40	
Late clinical sequelae	40 - 60	
Subjective symptoms	> 50	

Lead from exhaust gases of motor vehicles is alleged to present a serious health hazard, especially in impairing the mental development of children in urban areas, where pollution is supposedly greatest. Many countries are, therefore, adopting a policy of statutory control of the amount of lead in petrol with a view to the eventual elimination in the near future (60).

(d) Copper

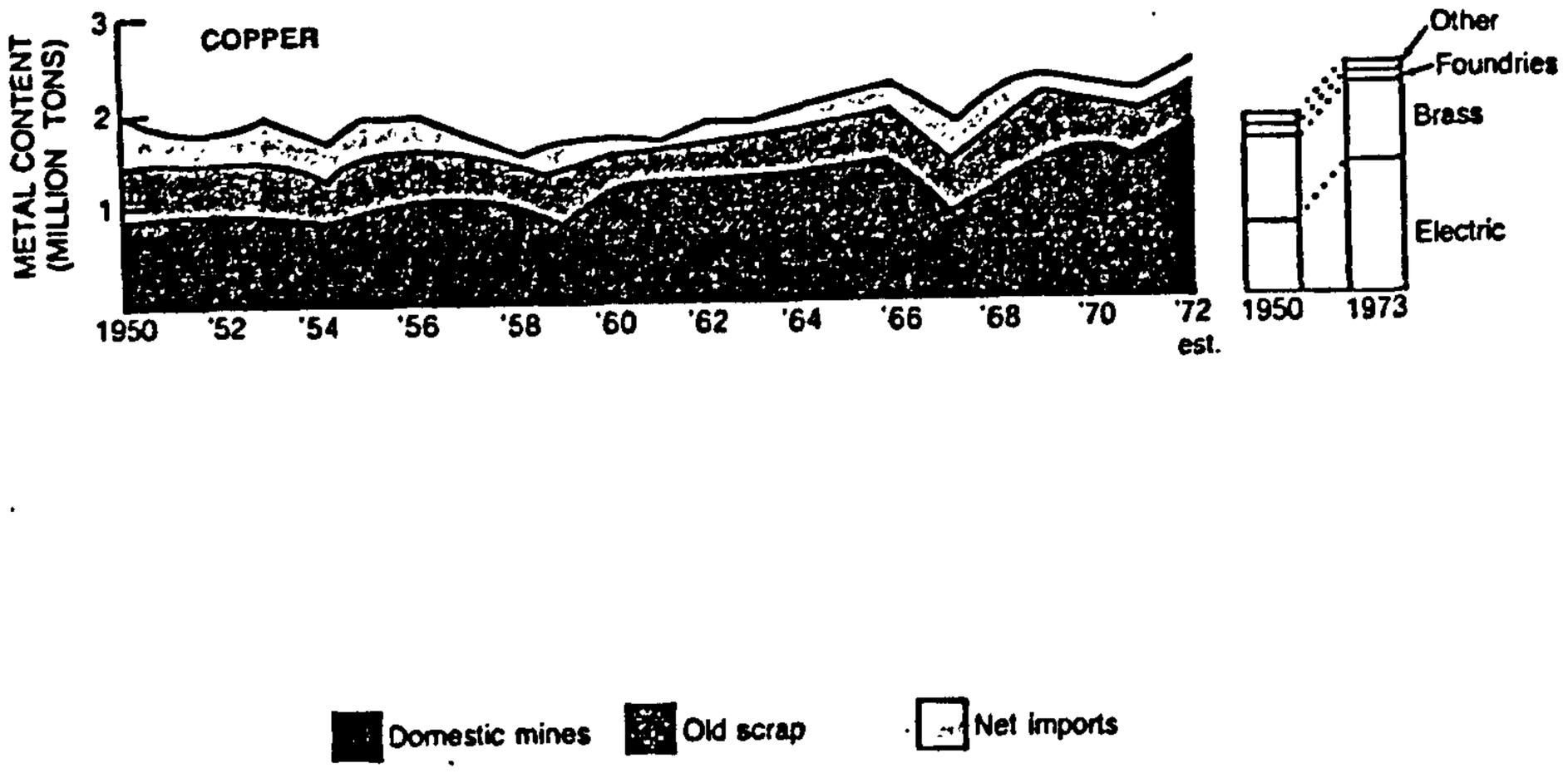
Copper occurs as the metal, but more often as a primary sulphide mineral (52). The principal copper ores are cuprite (Cu_2O), malachite ($CuCO_3Cu(OH)_2$), azurite ($CuCO_3$), chalcopyrite ($CuFeS_2$) and bornite (Cu_5FeS_4) (12). Clay and organic colloids (e.g. humic acids) are the major soil components involved in copper retention (61). The range of copper concentrations in soil is 2 to 250 mg/kg (8).

The most usual oxidation states are copper (I) and copper (II) (62) with Cu (I) being much softer than Cu (II) (63). Copper is an essential element, with a variety of functions playing an active role in a number of enzymes, for example, cytochrome oxidase, ascorbate oxidase, phenolase, laccase and diamine oxidase (64). In plants, copper performs key functions in respiration and photosynthesis. The element is an essential part of plastocyanin, an intermediate in the electron transport chain of photosynthesis. Copper proteins have been implicated in lignification, anabolic metabolism, cellular defense mechanisms and hormone metabolism (65).

The primary use of copper is in electrical equipment. It is also used in alloys (e.g. brass), chemical catalysts, anti-fouling paint, pesticides and wood preservatives, as well as for plumbing and heating (12).

The world mining production of copper for 1970 was 5.9 million tons, and on a smelter basis was 6.2 million tons. The major economic sources of copper are Canada, Peru and Chile (46). Usable world copper reserves amount to some 200 million tons (66). For the period 1950 to 1972, the supplies and uses of copper in the US are shown in Figure 1 (46).

Figure 1 : Supplies and uses of copper in US for 1950-1972



Sewage sludge has been found to contain between 92 and 3500 mg Cu/kg dry solids. Pesticides may cause severe local copper contamination (67,68). Copper mining, smelting and refining operations are major sources of copper pollution (31,69).

Much of the copper present in soils is in forms which are unavailable to plants (31). Five main soil copper fractions have been established in British soils :-

i) copper in soil solution and freely exchangeable copper

ii) copper weakly bound to inorganic sites

iii) organically bound copper

iv) copper occluded to free oxides

v) residual copper, held mainly in clay lattice structures (66).

Most of the copper is thought to be organically bound. In natural conditions, free copper ions are only rarely available for uptake from

the soil water solution. Little attention has been paid to the uptake of organo-copper complexes, but it is known that the charge of the applied copper complex is of great importance. Experiments with excised roots of Hordeum have shown that Cu-EDTA (negative) was poorly taken up, Cu-glycine (O) was taken up more readily and two (2+) complexes were taken up very readily. The chemical form of the applied copper is important in subsequent binding in the root. A considerable portion of root-absorbed copper (derived from $CuSO_4$ solution) was freely exchangeable with Ca^{2+} ions, whereas for both the (2+) complex bisethylenediamine copper and the (O) complex, copper glycine, a much smaller portion of the total root copper pool was exchanged. Thus the complex charge governs uptake, while the complex structure and, perhaps, stability governs subsequent binding (66,70).

The phytotoxic level of copper is over 20 mg/kg (40). A characteristic symptom of copper toxicity in plants is a reduction in chlorophyll content causing evenly distributed chlorosis over the leaf surface (31,72) as well as inhibition of root elongation (31).

In man, toxicity effects include vomiting, anaemia and jaundice. Damage to the nervous system, kidney and liver has been reported (14). Copper (Cu^{2+}) ions can induce destabilisation of the DNA structure (12,72). Deaths have been reported following ingestion of as little as 27 g of the salt, while others have recovered after ingestion of amounts up to 120 g. There is thus a marked variability in the manner in which individuals respond to a given exposure of the salt (14).

(2) Toxicity of heavy metals and the importance of speciation

The toxicity of heavy metals is not restricted to those metals thought to be non-essential (18,8,73). All elements, including the essential ones, are toxic at high concentrations (8). There exists a fairly narrow "concentration"window" between the essential and toxic levels (18). Toxicity leads to abnormal growth, disease or even death (8).

The potential toxicity of metals is controlled to a large extent by their physico-chemical form (18). The ligand preferences of trace elements can be summarised on the basis of the theory of hard and soft acids and bases (HSAB) (74,75). Metals can be identified as belonging to Class A, Class B or Class Borderline, as shown in Tables 11 and 12. In living cells, the important transition metals bind best to :

sulphur rich bases > nitrogen-oxygen bases > oxygen bases > coordination to water molecules

(74).

These metal-ligand reactions help to explain some of the toxic effects produced by metals and include the breaking of hydrogen bonds (12), displacement of other metals from a ligand site (7) and the change in tertiary structure of proteins leading to inhibition of catalytic activity (76,7,77). The attachment of metal ions to membrane structures can lead to inhibition of active transport or to the alteration in passive permeability properties of the membrane (76). The interactions of metals with ligands of nucleic acids potentially may affect both transcriptional and translocation processes (77) and possibly underlies the mutagenic and carcinogenic action of certain metals (12). For example, some lead compounds can produce chromosomal abnormalities (7), while cadmium compounds are suspected carcinogens of connective tissue, lungs and liver (76,14).

Table 11 : Classification of hard and soft acids and bases (31)

CLASS A

Metal ions referred to as "hard acids", and possess ligand or donor atom preference :- $F^- > Cl^- > Br^- > I^-$, and for metal-binding donor atoms in ligands :-

O > S ~ Se

N > As

O > N > S

Most stable complexes formed with ligands containing oxygen

CLASS B

"Soft acids", and have opposite preference sequence :-

$I^- > Br^- > Cl^- > F^-$, and

Se ~ S > O

As > N

S > N > O

Most stable complexes with ligands contàining nitrogen and sulphur-centres

CLASS BORDERLINE

Metal ions distinct from Class A, but increasing degrees of Class B characteristics in the sequence :-

$Mn^{2+} < Zn^{2+} < Ni^{2+} < Fe^{2+} \sim Co^{2+} < Cd^{2+} < Cu^{2+} < Pb^{2+}$

Borderline metals form complexes with ligands containing oxygen, nitrogen or sulphur.

Table 12 : HSAB Classification of Acids and Bases (30)

ACIDS

Hard (A)

H^+, Li^+, Na^+, K^+
Be^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Mn^{2+}

Al^{3+}, Sc^{3+}, Ga^{3+}, In^{3+}, La^{3+}
N^{3+}, Cl^{3+}, Gd^{3+}, Lu^{3+}
Si^{4+}, Ti^{4+}, Zr^{4+}, Th^{4+}, U^{4+}
Pu^{4+}, Ce^{3+}, Hf^{4+}, WO^{4+}, Sn^{4+}
UO_2^{2+}, $(CH_3)_3Sn^{2+}$, VO^{2+}, MoO^{3+}
$BeMe_2$, BF_2, $B(OR)_3$
$Al(CH_3)_3$, $AlCl_3$, AlH_3
RPO_2^+, $ROPO_2^+$
RSO_2^+, $ROSO_2^+$, SO_3
I^{7+}, I^{5+}, Cl^{7+}, Cr^{6+}
RCO^+, CO_2, NC^+
HX (hydrogen bonding molecules)

Soft (B)

Cu^+, Ag^+, Au^+, Tl^+, Hg^+
Pd^{2+}, Cd^{2+}, Pt^{2+}, Hg^{2+}, CH_3Hg^+,
$Co(CN)_5^{2-}$, Pt^{4+}, Te^{4+}
Tl^{3+}, $Tl(CH_3)_3$, BH_3, $Ga(CH_3)_3$
$GaCl_3$. GaI_3. $InCl_3$
I^+, Br^+, HO^+, RO^+

I_2, Br_2, ICN etc.
trinitrobenzene, etc.
chloranil, quinones, etc.
tetracyanoethylene, etc.
O, Cl, Br, I, N, $RO^{\cdot}$, $RO_2^{\cdot}$
M^o (metal atoms)
bulk metals
CH_2, carbenes

Borderline

Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Sn^{2+}, Sb^{2+}, Bi^{3+}, Rh^{3+}, Ir^{3+}, $B(CH_3)_3$,
SO_2, NO^+, Ru^{2+}, Os^{2+}, R_3C^+, $C_6H_5^+$, GaH_3

BASES

Hard

H_2O, OH^-, F^-
$CH_3CO_2^-$, PO_4^{3-}, SO_4^{2-}
Cl^-, CO_3^{2-}, ClO_4^-, NO_3^-
ROH, RO^-, R_2O
NH_3, RNH_2, N_2H_4

Soft

R_2S, RSH, RS^-
I^-, SCN^-, $S_2O_3^{2-}$
R_3P, R_3As, $(RO)_3P$
CN^-, RNC, CO
C_2H_4, C_6H_6
H^-, R^-

Borderline

$C_6H_5NH_2$, C_5H_5N, N_3^-, Br^-, NO_2^-, SO_3^{2-}, N_2

(The symbol R stands for an alkyl group such as CH_3 or C_2H_5).

Information on the chemical speciation of a trace element in the environment is essential for an adequate understanding of biogeochemical behaviour and of significance to human health (78). Toxicity is markedly dependent on the speciation of the material concerned (79). For example, tetraethyl lead is readily absorbed from the respiratory tract, gastrointestinal tract and skin, whereas the insoluble and stable lead sulphate, sulphide and chromate are poorly absorbed from the gastrointestinal tract (76). The determination of total metals (extractable by strong acids) in the environment indicates the extent of contamination, but not its significance (19).

In natural waters the toxicity of a metal ion and the rate of uptake by organisms is predominantly controlled by the concentration of the "free", hydrated ion, and not by total concentration (1). For example, the toxicity of solutions containing zinc is mainly attributable to the zinc ion and perhaps also to particulate zinc present as the basic carbonate or the hydroxide held in suspension (80). The toxicity of a particular dissolved metal species is probably related to its ability to react with a biological membrane. Penetration of the membrane by a metal ion depends on the direct lipid-solubility of the metal species (usually only as an uncharged organometallic species), or the extent and rate of reaction of the metal ion with a membrane transport protein (18). The effect is demonstrated by the different effects produced by ionic and organic lead. Inorganic lead does not give rise to the nervous effects produced by organic lead. The toxicity to the central nervous system of organolead is undoubtedly associated with rapid penetration of the blood-brain barrier (12).

Investigations have indicated the importance of speciation in plant uptake of heavy metals (24,23,81,82). Brown et al. found the total copper levels in soils were of little diagnostic value, but extraction

of organically complexed copper with EDTA could be used to indicate copper availability (83). Accumulation of a given element appears to be largely determined by its solubility in soil solution (24). The availability of heavy metals to microorganisms and plants in the soil-water system depends on a number of interacting factors particularly :-

i) inorganic and organic complexing agents

ii) absorption onto clays and inorganic matter

iii) metal-metal interactions

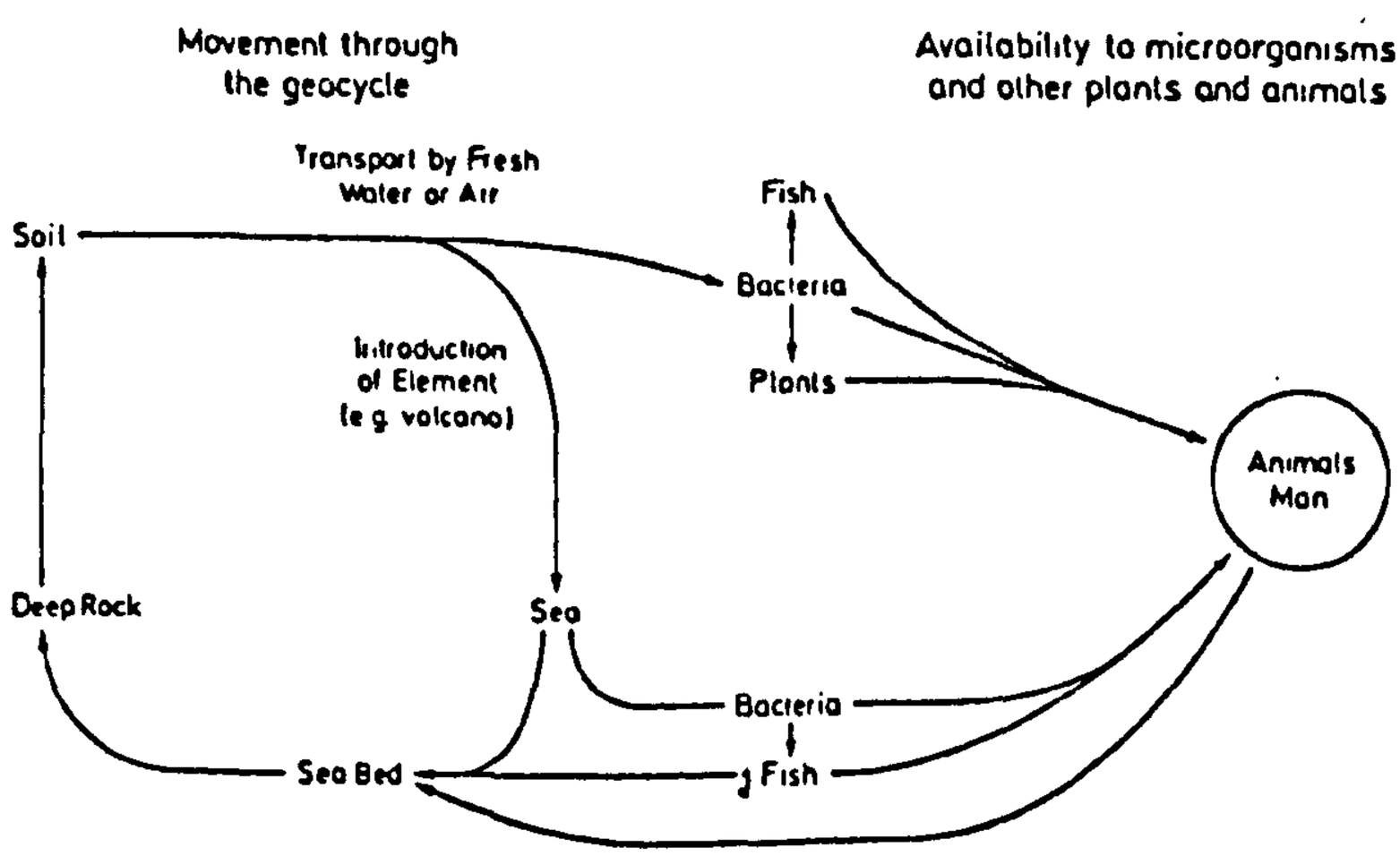
iv) formation of sparingly soluble salts (82).

The total concentrations of metals in soils, their chemical forms, mobility and availability to the food chain, provide the basis for a range of problems in crop, animal and human health (81).

3) Associations and transport of heavy metals

Static equilibria are very rare on the earth's crust. The hydrosphere, lithosphere, atmosphere and biosphere are in dynamic equilibrium - characterised by inputs, outputs and residence times for its constituents (84). Movement through the biogeocycle is shown in Figure 2 (85).

Figure 2 : Movement of toxic elements through the geocycle



A knowledge of heavy metal speciation is necessary in order to understand their behaviour and transport through the hydro/geo/biosphere (86).

Heavy metals are present in many different chemical forms, which may be broadly classified into three groups :-

i) inorganic compounds

ii) simple and inorganically complexed ions

iii) metal organic compounds.

The metal-inorganic compounds in the environment are generally only present in solid phases, although these solid phases may exist in colloidal suspension in natural waters (85). The possible physico-chemical forms of metals in natural waters are indicated in Table 13 (18).

Table 13 : Possible physico-chemical forms of metals in natural waters

Physico-chemical form	Possible examples	Approximate diameter, nm
Particulate	Retained by 0.45 μm filter	> 450
Simple hydrated metal ions	$Cd(H_2O)_6^{2+}$	0.8
Simple inorganic complexes	$Pb(H_2O)_4Cl_2$	1
Simple organic complexes	Cu-glycinate	1-2
Stable inorganic complexes	PbS, $ZnCO_3$	1-2
Stable organic complexes	Cu-fulvate	2-4
Adsorbed on inorganic colloids	Cu^{2+}-Fe_2O_3, Pb^{2+}-MnO_2	10-500
Adsorbed on organic colloids	Cu^{2+} - humic acid	10-500
Adsorbed on mixed organic/ inorganic colloids	Cu^{2+} - humic acid/Fe_2O_3	10-500

The transport of metals across biological membranes requires that the metal be in solution. Therefore, elements in solid phases must normally be converted to ionic or organically combined form in solution before they can enter the biosphere (87). Thus the biogenous removal of metals from the water column is speciation dependent, in the sense that the extent of metal uptake by planktonic organisms and adsorption to sinking detritus depends on the forms of metal present (88).

Studies of river, estuarine and marine sediments have shown that a number of metals are concentrated in sedimentary material. Removal of many trace elements is known to be related to adsorption or other surface association phenomena with hydrous metal oxides, clays, or detrital organic matter (89). However, once the metal reaches the sediment it is not necessarily fixed there for all time, but may be recycled via biological and chemical agents, both within the sediment and also back into the water column (11).

In general, metals in aquatic sediments can be divided into those that are relatively immobile (in the long term) and those whose ease of mobility (in the short term) poses some importance as far as exposure to the biosphere is concerned (90). Such a division also applies to soils. Trace elements may be present in soils in :-

1. primary and secondary minerals
2. as precipitated salts
3. in ionic and complexed state in the soil solution
4. complexed by organic materials
5. adsorbed onto reactive surfaces of soil constituents (81).

The availability of metals and thus uptake by plants is related to their total concentrations, forms and associations in the soil as well as a number of physical and chemical factors operating at the soil root surface (81).

Clay minerals (11), organic matter (8,91) and hydrous oxides (92) all play important roles in the transport of metals through the hydrogeosphere (89).

a) Clays

Clays are aluminosilicates with layer structures, many of which swell in water and function as cation-exchangers (8). They are major components of many soils and sediments and the suspended matter in streams. Their plate-like particles range in size from a few hundredths of a micron to several microns (93).

The ability of clay minerals to take up metal ions from metal-salt solutions is well documented (11). The order of affinity of cations for clay minerals is determined mainly by their charge and to a lesser extent by their size (8). Studies have indicated that if the cation exchange capacity was fully saturated, kaolinites might contain 300 µg/g of zinc and montmorillonites about 30,000 µg/g. However, naturally occurring clays usually hold far less zinc which is probably due in part to the presence of much higher concentrations of cations such as Ca^{2+} which will compete with heavy metals for the cation exchange sites. Thus, despite their potential for cation exchange, clays are not necessarily the main reservoir for heavy metals in soils or sediments (11).

The mixing that occurs between organic matter, iron oxides and clay particles renders the role of clay minerals in heavy metal uptake difficult to evaluate, all three components being enriched in the "clay-size" or pelitic fractions of sediments (11).

b) Organic matter

Naturally occurring organic complexants affect heavy metal behaviour in soils, natural waters, and other parts of ecosystems.

They are abundant in most soils, highly diverse in their chemical nature, but may be broadly divided into two groups :

i) non-humic substances

ii) humic substances.

Non-humic substances are compounds which have recognisable chemical characteristics e.g. carbohydrates, proteins, amino acids, fats, waxes, resins and pigments. Due to their susceptibility to microbial degradation, these compounds have a limited lifetime in soils (96).

Humic substances are the major organic constituents of soils and sediments, occurring in almost all terrestrial and aquatic environments. They arise from the chemical and biological degradation of plant and animal residues and from synthetic activities of micro-organisms.

Humic substances are dark-coloured, acidic, predominantly aromatic, hydrophilic, chemically complex, poly-electrolyte-like materials that range in molecular weight from a few hundred to several thousand daltons. They are usually divided into three main fractions, on the basis of their behaviour in acid and alkali :-

a) humic acids - soluble in dilute alkali, but precipitated on acidification

b) fulvic acids - remains in solution, when the alkali-extract is acidified

c) humin - not extractable with either dilute base or acid.

Structurally these three fractions are similar, but differ in molecular weight. Fulvic acid has the lowest molecular weight, contains more oxygen, but less carbon and nitrogen with a higher content of oxygen-containing functional groups per unit weight than the other two humic fractions (95). The detailed structure of these fractions remains controversial, but is thought to consist of an aromatic core

bearing, as main substituents, carboxylic and phenolic groups (96,91).

Humic substances are capable of interacting with metal ions, metal oxides, metal hydroxides and more complex minerals to form metal-organic associations of widely differing chemical and biological stabilities and hence characteristics. Humic materials contain per unit weight relatively large numbers of oxygen-containing functional groups (CO_2H, phenolic OH, C=O), through which they can attack and degrade soil minerals by complexing and dissolving metals and transporting these within soils and waters.

Although the mechanism by which humic substances bind metal ions remains speculative (91), the complexing properties are also attributed to the phenoxy and carboxyl functional groups (97). The fact that metal complexation by humic substances results in the release of protons indicates to some extent that the same ligands are involved in proton and metal binding (98). Experiments to determine the complexing behaviour of cadmium, copper, lead and zinc indicate that complexing increases with increasing pH, except for zinc and cadmium below pH 6. At pH 4.5 increasing ionic strength of the solution does not affect the formation of metallo-organic complexes significantly for copper and lead, but does for cadmium and zinc (99). Either one acidic CO_2H and one phenolic OH group or two acidic CO_2H groups, in both instances in close proximity, reacted simultaneously with metal ions and hydrous oxides.

Due to the complex structure of these naturally occurring poly-electrolytes, it is conceivable that, depending on the nature and position of substituents in aromatic rings, different structural units can be found. Thus, dissimilar complexation mechanisms could occur resulting in heterogeneous binding of metal ions (96).

(c) Hydrous oxides

Hydrous oxides have long been regarded as playing an important role in the binding of trace metals, soils and sediments (100). Indeed, Jenne considered them to be capable of playing a dominant role in controlling the concentrations of metal ions (Mn, Fe, Co, Ni, Cu, Zn) in soils and waters (92). This role is due in part to their adsorptive capacity (101,93) in part to the fact that formation or dissolution of such substances can be induced by changes in pH, oxidation potential or the presence of complexing agents (93).

The hydrous oxides of aluminium, iron and manganese are amorphous in character (101) and have limited ion-exchange properties, as well as acting as specific absorbents for phosphate and some of the rare elements (8).

The sorption or desorption of heavy metals occurs in response to :-

i) the solution concentration of the metal in question

ii) the concentration of competing metals

iii) hydrogen ion concentrations

iv) formation and destruction of organic chelates and inorganic complexes.

In general, there is a dramatic increase in the adsorption of foreign ions on hydrous oxides with increasing pH, while hydrogen ions are released into solution. The adsorption and in turn, release of hydrogen ions is dependent on the surface area of hydrous oxide (100). In some acid soils Al^{3+} and Mn^{2+} may become important exchangeable cations, as some of the hydrous oxides begin to dissolve at pHs lower than 4 (8).

Due to their ability to exist as partial coatings on silicate minerals rather than as discrete, well-crystallised minerals, the oxides in soils and recent sediments exert a chemical activity which is far

out of proportion to their activity (92). Colloidal hydrous iron oxide surfaces can retain a positive charge up to a pH of 8, so that some degree of interaction between the oxide negatively charged clay surfaces is to be expected. Formation of larger particles through coagulation is apparently minimal, and sorption on smaller particles appears to be favoured. The intrinsic affinities of the heavy metals for the oxide surface decrease in the order Cu > Pb > Zn > Co > Cd (93).

Attempts have been made to distinguish between the metal binding capacities of the iron-rich and manganese-rich phases of ferromanganese deposits. But the situation is not clear. In soils, it has been concluded that lead was concentrated in manganese oxides whereas zinc was present at similar levels in both manganese and iron oxides (11).

Where streams flow across mineralised zones, zinc concentrations in ferromanganese oxide coatings have been observed to increase from about 100 to over 2000 ug/g. Hydrous oxides are evidently effective scavengers of some soluble metals, particularly zinc, probably cadmium and sometimes lead (11). Jenne emphasised their potential role in removing metals from contaminated waters to the underlying sediments (92), and this has been confirmed in the case of acid mine drainage.

The pH sensitivity and reversible nature of the adsorption of heavy metals is of great environmental significance. Large seasonal fluctuations in pH can be encountered in poorly buffered natural water systems (100) and although soils are generally buffered within a moderately narrow pH range, extremely acid conditions can arise for example when pyrites are oxidised by dissolved oxygen in mine runoff fluids (100).

4) Heavy metals in freshwater and sediments

Heavy metals may exist as a number of different chemical entities in natural waters. Different species can be distinguished in solution and the solid phase. The following may be present in the dissolved state :-

1. as hydrated ions
2. complexed with inorganic ligands, such as chloro, carbonato, and hydroxo complexes, i.e. more or less labile complexes
3. chelated in rather stable and in inert complexes with organic ligands such as amino, fulvic, humic and nucleic acids, proteins, and chelating agents of anthropogenic origin (102).

In the solid phase metals can be found associated with colloidal particles, or adsorbed at or incorporated in suspended matter or sediments. The suspended and colloidal particles may consist of individual or mixed hydroxides, oxides, silicates, sulphides, or other compounds, or they may consist of clay, silica, or organic matter to which metals are bound by adsorption, ion exchange, or surface complexation (102,103).

Trace metals reach the sediment in three principal ways :-

i) in or on particles which settle to the bottom

ii) in or on particles which are transported along the bottom

iii) by the sorption of dissolved metals from waters in contact with the sediment.

The sedimentation of particles is usually the most important route to the bottom sediments. Three classes of particles may be distinguished: detrital, biogenous and precipitated :-

1) Detrital particles - heavy metals contained within the crystal lattice, adsorbed on the surface, in exchange sites on clay-type

minerals, and in surface coatings formed by hydrous oxides or organic matter.

2) Biogenous particles - heavy metals contained within inorganic skeletal material, adsorbed on surfaces, in exchange sites of organic materials, complexed to organic matter, and in coatings of hydrous oxides which may form on particles.

3) Precipitated particles - e.g. calcium carbonates, hydrous oxides and sulphides which carry heavy metals adsorbed on their surface, as co-precipitated material, and as metal compounds precipitated as discrete particles.

As such particles settle through the water column they may be partially dissolved by bacterial attack or by changes in the chemical environment. In addition to physical or hydraulic factors there are several chemical factors which influence the depositional environment; thus Eh, pH, salinity, temperature, nature and amount of organic matter are the most important (11,104).

In general, sediments are deposited under either oxidising or reducing conditions. Brownish sediments containing iron oxides are indicative of a fully aerated environment, whereas grey or black sediments containing iron sulphides signify a reducing medium (11).

Under natural conditions heavy metal levels in river sediments reflect their occurrence and abundance in rocks and mineral deposits in the river catchment area. In many cases man-made contributions of heavy metals to river sediments have equalled or exceeded the amounts released by weathering (105).

Metals discharged in particulate form reach the sediment directly. Those discharged in solution reach the sediment indirectly as described above. Thus, if the metal concentration in the water column remains consistently high, the associated sediments will nearly always be highly

contaminated. Lead, in particular, is carried as a suspended solid rather than in solution (11). On the other hand, copper is more likely to react with aquatic organic ligands (106). Cadmium, copper, lead and zinc levels in freshwater and sediment are shown below (8).

Table 14: Median levels of Cd, Cu, Pb and Zn in freshwater and sediment

1. Freshwater

METAL X	MEDIAN CONCENTRATION µg X/l	RANGE µg X/l	MAJOR SPECIES
Cd	0.1	0.01 - 3.00	ORGANIC
Cu	3.0	0.20 - 30.00	ORGANIC
Pb	3.0	0.06 - 120.00	NOT KNOWN
Zn	15.0	0.20 - 100.00	Zn^{2+}, ORGANIC

2. Sediment

METAL X	MEAN SEDIMENT mg X/kg
Cd	0.17
Cu	33.00
Pb	19.00
Zn	95.00

Sediments vary enormously in their average particle size and in the range of sizes present. The finer fractions (2 µm diameter, i.e. "clay" or "pelitic" size fraction) contain not only a larger proportion of clay, iron and organic matter than the coarser fractions (the "silt", 2-50 µm, and "sand", > 50 µm size fractions), but also a higher concentration of trace elements such as lead and zinc (11).

Resuspended sediments have been found to be capable of extracting Cu, Pb, Zn, Cd, Ni and Co from 100 ppm aqueous solutions. The absorption was strong for Cu, Pb and Cd, moderate for Zn and weak for Ni and Co. Absorption was found to be enhanced by high pH, warm water and for Co and Zn darkness (107).

The load of suspended matter rises following heavy rains when particles from soils and sediments are mobilised. Near mines and smelters and in urban areas this can lead to large but temporary increases in the levels of suspended metals (11).

5) Heavy metals in soils

A soil may be regarded as a multicomponent, reactive system comprising solid, liquid and gaseous matter (108). Because one of the main pathways of metals into human and animal bodies is through the food chain, there is considerable concern about metal uptake by plants from soils (13,109). Livestock also involuntarily ingest appreciable quantities of soil - in cattle the daily intake ranges from 140 to 1400 g soil/day (110).

Metals in soils are derived either from natural geological sources or from the activities of man (13). Lead, copper and zinc contents of 25, 15 and 20 mg/kg may be considered as normal (111) and for cadmium most soils contain very small amounts of less than 1 mg/kg (112). However, soils in several parts of Britain have accumulated considerable amounts of heavy metals, arising largely from activity by man (113). For example, at Shipham in Somerset calamine mining in the past has caused contamination of the soil - up to 800 mg Cd/kg and 6.2% Zn. At Avonmouth, Avon, zinc, lead and cadmium contamination has taken place in the vicinity of the lead-zinc smelter (44).

The soil may be regarded as a natural reservoir for the contaminating

metals (114). The availability of heavy metals to microorganisms and plants in soils depends on a number of factors (82). The major factor is the solubility and the thermodynamic activity of the uncomplexed ion, since in order for root uptake to occur, a soluble species must exist adjacent to the root membrane for some finite period. The rate and extent of solubilisation are governed by the physicochemical properties of the deposited material, soil processes, and soil properties. The soil physicochemical parameters most important in influencing the solubility of metals include :-

i) solution composition (inorganic and organic solubles)
ii) Eh and pH
iii) type and density of charge on soil colloids
iv) reactive surface area.

These phenomena will be dependent upon soil properties, including :-

i) metal concentration and forms
ii) particle size distribution
iii) quantity and reactivity of hydrous oxides
iv) mineralogy
v) degree of aeration
vi) microbial activity (115).

The total concentration of heavy metals in the soils may indicate the potential toxicity, but the proportion of such metals in various chemical forms will determine the actual toxicity in the soil-plant system at a particular point in time (113).

Soil zinc may be :-

i) in solution as ionic or organically complexed species
ii) on exchange sites of reactive soil components
iii) complexed with organic matter
iv) occluded in oxides and hydroxides of Al, Fe and Mn

v) entrapped in primary and secondary minerals (116).

Zinc mobility and thus availability decreases with rising soil pH (81).

Cadmium is relatively mobile in some soils and may be redistributed due to leaching down the profile (81). John et al. found that cadmium accumulation occurred in the surface layer, its level progressively decreasing with depth, thus indicating that downward movement of cadmium in the soil profile may occur at a limited rate (117). Recent reports suggest that when metallic cadmium and divalent cadmium ions are associated with phosphate and carbonate anions their leaching should be limited (118). Esser and El Bassam found only a fraction of the total cadmium could be regarded as being bound strongly to organic substances (82). Major influences on the availability of cadmium are believed to be soil organic matter, clay content, pH, and redox potential.

Lead deposited on the soil surface does not usually leach down the profile, probably because of the strong absorption of Pb^{2+} on the surface of clay minerals, organic colloids and to the formation of insoluble lead chelates with organic matter (81).

Byrd et al. reported that lead concentration decreased with depth but began to increase when a heavy blue clay was encountered, indicating lead absorption by the clay (119).

The low solubilities of lead phosphates suggest that their formation may be of crucial importance in the dispersion and fixation of lead (and phosphor us) in ecosystems (56).

Lead mobility in soil containing an organic-rich top layer is influenced by the presence of humic acids. Lead is absorbed by humic acids and thus becomes concentrated in humus-rich material (51).

Copper in the soil is strongly held on inorganic and organic sites and in complexes with organic matter. As such, a large proportion of the total copper content of soils is not available for uptake by plants

(81). Thus, copper is relatively immobile in soils. Studies have indicated that clays, organic matter and hydrous oxides(particularly those of Mn) all provide strong sorption sites for copper (70).

The mobility of copper in biological systems is determined to a very large extent by its coordination chemistry; copper often forms stronger complexes with many organic ligands than other divalent transition metal ions (5 & 70). The largest fraction of soil copper is that associated with organic matter (70).

Copper toxicity commonly occurs on very acid (below a pH of 5) soils with low cation exchange capacities (120). As the pH is adjusted toward neutrality the mobility of copper is reduced as a result of increased sorption onto mineral surfaces (70).

The soil factors influencing the concentration, form and plant availability of metals are highly complex, and use of plants as monitors of increased metal levels arising from pollutant sources is clearly dependent upon an understanding of these phenomena (115).

6) Heavy metals in plants

The plant uptake of chemical species from soil solution is dependent on a number of plant/soil interactions. These include :-

i) physical processes such as root intrusion

ii) water and ion fluxes and their relationship to the kinetics of metal solubilisation in soils

iii) biological parameters, including kinetics of membrane transport, ion interactions, and metabolic fate of absorbed ions; and the ability of plants to adapt metabolically to changing metabolic stresses in the environment.

The process of root intrusion within the soil profile provides an extensive rhizosphere for ion absorption, but the concentration of

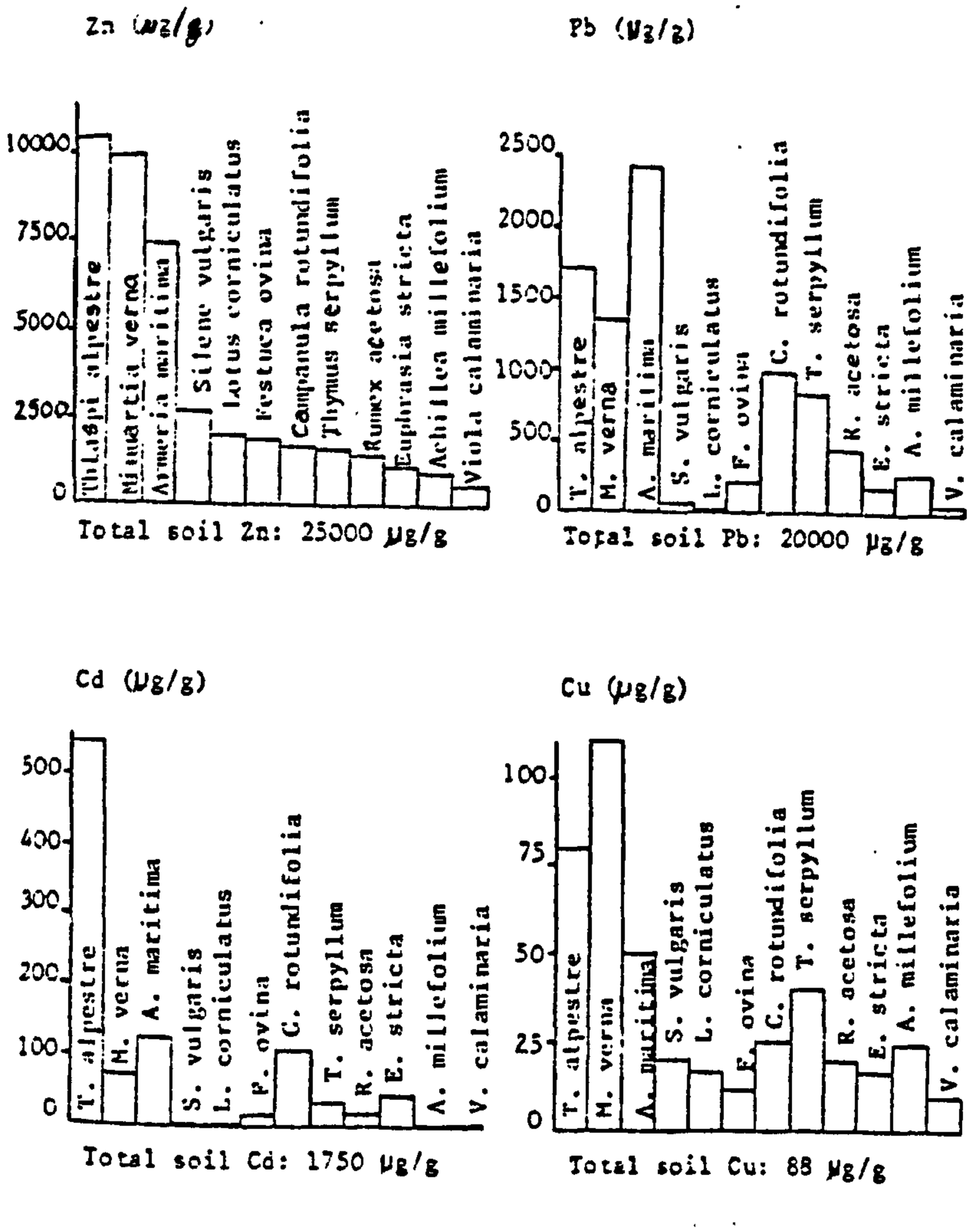
individual ions in solution can be rapidly depleted by plant uptake. Depletion of ions in the rhizosphere is alleviated to some extent by diffusion of ions, and by mass flow of both water and ions from surrounding soil induced by the transpirational demand of the plant. Ultimately, the supply of ions within the rhizosphere is controlled by the kinetics of solubilisation of ions sorbed to the solid phase of soil, and the kinetics of removal by the plant root. In effect, the elemental composition of the plant reflects (to some extent) the composition of the soil solution (115). The background levels of cadmium, copper, lead and zinc in some plants are given in Table 15 (8).

Table 15 : Background levels of Cd, Cu, Pb and Zn of some plant types in mg X/kg DM

X	Bryophytes	Woody gymnosperms	Woody angiosperms	Herbaceous vegetables
Cd	0.1 - 1.2	0.1 - 0.9	0.02 - 2.4	0.05 - 0.09
Cu	6 - 40	5 - 13	6 - 14	4 - 20
Pb	8 - 280	0.9 - 13	1 - 8	0.2 - 20
Zn	12 - 250	20 - 90	34 - 68	14 - 160

Figure 3 shows the levels of zinc, lead, cadmium and copper in a range of species growing on naturally metalliferous soil (121).

Figure 3 : Concentrations of lead, zinc, cadmium and copper in the leaf dry matter of a range of species growing on a naturally metalliferous soil in Germany



Studies have indicated that abiotic and biotic soil processes control the solubility and availability of metals for plant uptake. Metals were taken up by plants at differing rates, and, once absorbed, their mobility within the plant varied, suggesting a second point of

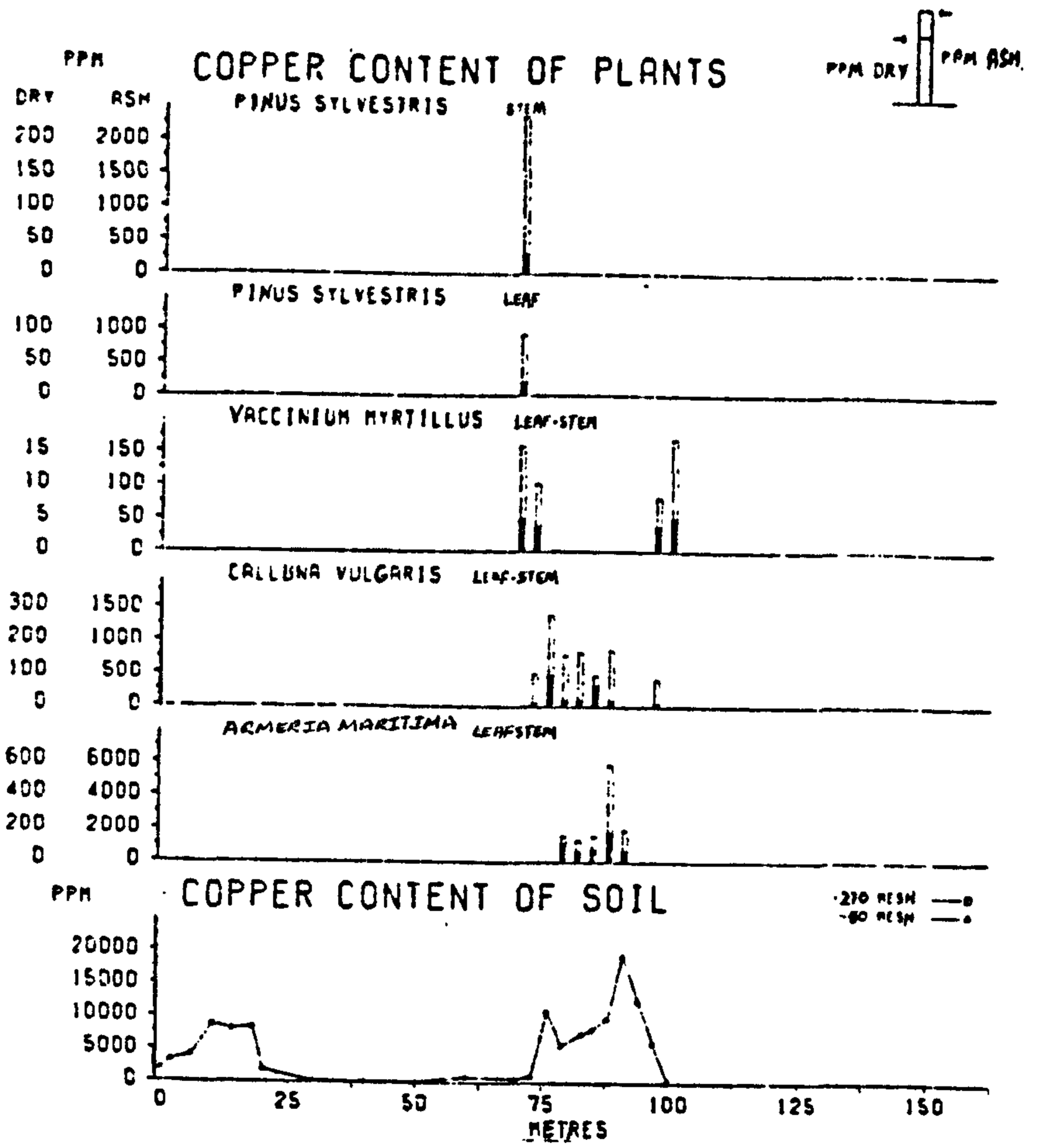
metabolic regulation (115). In addition, variation in metal uptake differs between plant species and varieties. For example, lettuce and tomato showed increase in cadmium contents in various parts of the plant when grown on soil supplemented with cadmium, whereas no such accumulation of the metal was found in potato or string beans (112). Considerable variation of metal levels in different tissues of plants has also been observed. For example, Jones et al. reported that movement of lead to the tops of actively growing ryegrass is restricted by the roots (122). Chino and Baba found that metals of high electronegativity such as Cd and Ni were mainly accumulated in the roots of rice seedlings and only a small quantity was translocated to the tops (23).

Some plants may be described as being metal tolerant (Figure 3), which refers to either 1) any species found occurring in an area of toxicity from which other species appear to be excluded, or 2) to specific individuals of one species which are able to withstand greater amounts of toxicity than other individuals of that species on normal soil (27).

Plants known to be tolerant to high concentrations of metals in soils have been used in biogeochemical prospecting, their presence indicating high concentrations of precious metals in soils (19). One of the most studied indicator plants is Becium homblei (123) which is able to tolerate soil copper concentrations of up to 15 000 mg/kg and soil nickel concentrations of nearly 5000 mg/kg (124). Another example of an indicator plant is Armeria maritima, a species which can be found associated with the Coed-y-Brenin porphyry copper deposit (containing 0.2% copper), North Wales (125). Figure 4 indicates the copper content of the soil and some associated plant species (126).

Other examples include Polycarpaea glabra and Eriachne mucronata associated with lead-zinc deposits (125).

Figure 4 : Dolfrwynog Bog : Transect showing analyses of soil and plants for copper content



It appears that the plant species that occupy environments contaminated with heavy metals only do so because they evolve metal tolerant populations. The evolution of tolerance can be very rapid. The species that evolve tolerance appear to be those that contain genes for tolerance in their normal populations. The occurrence of species in contaminated areas is therefore determined by evolutionary ability rather than innate physiological tolerance (127).

Metal tolerant plant populations can be used to reclaim metalliferous

wastes (127-130). Such wastes are usually unstable and subject to wind and water erosion, leading to extensive contamination of the locality (128,130). Seed from the most tolerant populations of the grasses Festuca rubra and Agrostis tenuis has been developed on a commercial scale by the National Seed Development Organisation (128), and the following varieties are now available (127) :

Merlin	Red fescue (Festuca rubra)
	for neutral and calcareous lead/zinc wastes
Goginan	Bent grass (Agrostis tenuis)
	for neutral and acidic lead/zinc wastes
Parys	Bent grass (Agrostis tenuis)
	for neutral and acidic copper wastes.

Heavy metal levels in the sward often reach 5000 ug/g Pb, 1000 ug/g Zn and 500 ug/g Cu. These concentrations are too high for the swards to be continuously grazed (128).

The evolution of metal tolerance has also been found in bacteria and fungi. Bacterial resistance to heavy metals can result in bio-accumulation, biotransformation, changes in ecological diversity and co-selection of resistance factors for antibiotics (131). The use of metal containing pesticides and antifouling paints has led to the evolution of tolerant varieties. For example, the leaf rust Hemilia vasatrix can tolerate exceedingly high copper concentrations on the surfaces and in the tissues of its host Coffea arabica, the bark of which can contain over 750 ug/g of copper (68). However, forest litter and soil microbial communities which regulate the immobilisation and release of essential elements can be adversely affected by heavy metal contamination (132), which also affects decomposition rates (132-135).

Heavy metal enrichment of vegetation and leaf litter due to aerial

contamination has been investigated (2, 135-138). Airborne heavy metals fall upon, react with and are absorbed by plants and soils near the source of pollutant generation (136). The surface deposited metals may be absorbed by the vegetation, but the physiological effects are not clearly defined (138). Some metal particulates may enter directly through the stomatal apertures (37). Little and Martin suggest that lead remains largely as a superficial deposit on the leaf, where much of it can be removed by washing, whereas zinc and cadmium show at least partial penetration of the leaf (137).

The evolution of heavy metal tolerance due to aerial fallout of metal particulates has been demonstrated (69,139,140).

Three types of plant-soil relationship have been proposed, where plants are described as (121,141) :-

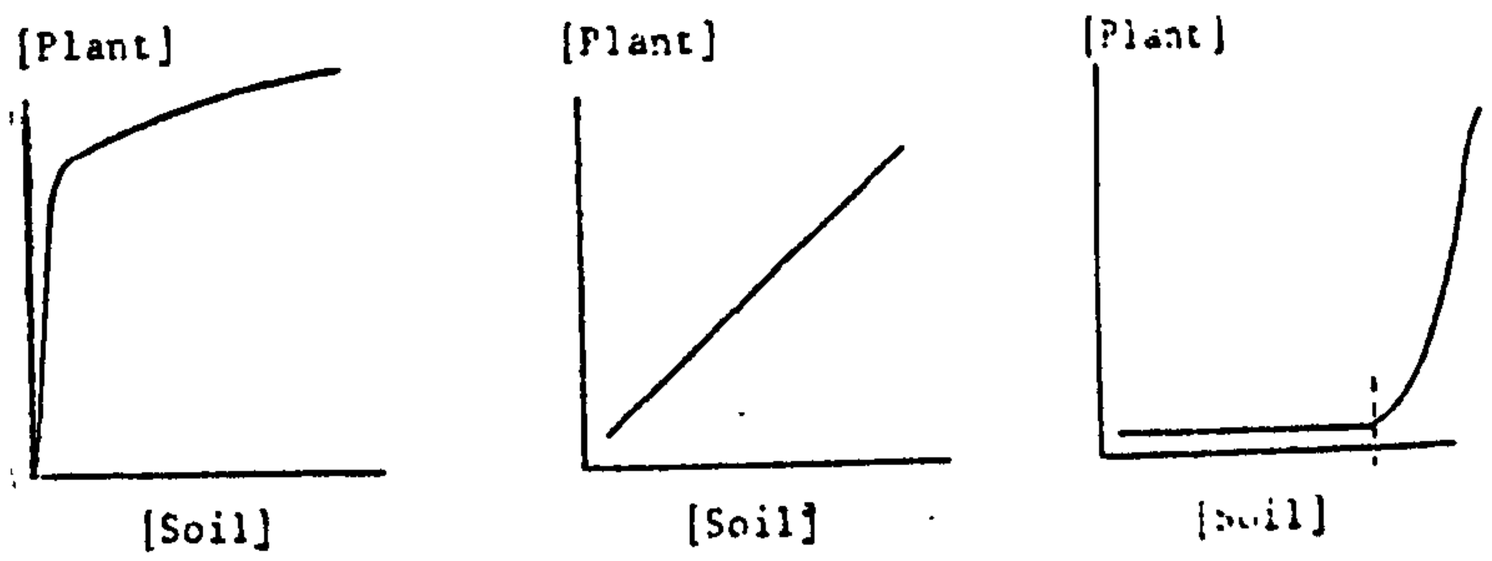
1. Accumulators - these plants can be defined as concentrating metals within their above ground plant parts from low or high soil levels. In certain cases the term hyperaccumulator is used if the plant exceeds 1000 λug metal/g dry plant weight.

2. Index plants - plants whose internal concentration reflects external levels.

3. Excluders - or plants with restricted transport. Metal concentrations in the shoot are maintained constant but low over a wide range of soil concentration, up to a critical soil value above which the mechanism breaks down and unrestricted transport results (121,141).

These 3 plant-soil relationships are demonstrated in Figure 5 (121).

Figure 5 : The response of plants to increasing soil metal levels



Accumulator Index Excluder

Table 16 indicates the high metal levels that may be found in some plants.

Table 16

Plant species	metal and concentration	Ref.
Se bertia ac uminata	10 000 mg Ni/kg	19
Thlaspi alpestre	7 757 ug Zn/g D.W.	27
Armeria maritima (ssp) halleri	3 328 ug Zn/g D.W.	27
Viola calaminaria	686 ug Zn/g D.W.	27
Haumaniastum robertii	1.02% Co & > 0.2% Cu D.W.	123
Sutera fodina	48 000 ug Cr/g Ash	141

Heavy metal tolerance in plants is a well documented but poorly understood phenomenon (142,140). Over the past several years, studies on heavy metal tolerance have become more broadly based, which has

resulted in the accumulation of information along a wider front, rather than a marked advancement in any one particular area (143). Until recently, tolerance in plants from areas affected by aerial contamination had not been studied (139).

At the present time, a clear-cut picture of what types of mechanism are operating in ecotypes which are tolerant to various heavy metals is not available (143). The situation is confused by the fact that no definite taxonomic pattern has emerged; the species found on toxic soils being very varied and differing according to the local ecological conditions and geographic area. The situation is further complicated, since the existence of a species on a metal contaminated site may not be due entirely to its ability to tolerate high metal concentrations, but may also, in part, be due to the plant's ability to tolerate extremely low concentrations of essential nutrients, such as nitrogen, phosphor us and potassium, or high concentrations of others, such as sulphur (27).

The suggested mechanisms of tolerance to heavy metals include (27) :-

i) Restricted transport within the plant

ii) Removal of metal ions by rendering into an innocuous form, e.g. binding with a protein, organic acid or cell wall

iii) Adaptation of the plasmalemma

iv) Enzymic adaptations

v) Removal of metal ions from metabolism by pumping from the cell

vi) Removal of metal ions from metabolism by deposition in the vacuole.

These possible mechanisms of heavy metal tolerance are outlined below :-

Exclusion/restricted transport

Evidence suggests that plants growing on toxic metalliferous soils

cannot prevent metal uptake but only restrict it and hence accumulate metals in their tissues to varying degrees (121,141,143). There are a number of examples of metals being restricted to the roots. Copper in copper tolerant and non-tolerant Agrostis stolonifera accumulated only in the roots of the tolerant race, but passed into the shoot of the non-tolerant plants (143). Similar results were reported for Zn/Pb tolerant and non-tolerant races of Silene maritima (121). Jones, Clement and Hopper concluded that the roots of actively growing ryegrass provide a barrier which restrict the movement of lead to the above ground parts of plants (144). Oat (Avena sativa) roots in contact with contaminated soil accumulated large amounts of cadmium (mean 205.1 µg/g) whereas the shoots contained a much smaller concentration (mean 16 µg/g) (117). However, restricted transport has not been found in other species, for example, zinc-tolerant clones of Agrostis tenuis, Agrostis stolonifera and Deschampsia caespitosa all accumulate as much zinc in their roots and shoots as do non-tolerant clones (131), while radish plants (Raphanus sativus) accumulate more cadmium in the tops than in the root portion (6).

Thus, although some plants show restricted transport of the metal to the shoot, it cannot be a universal mechanism for all species and metals (143).

Role of the cell wall/cell membrane

The analysis of subcellular fractions of the roots of metal tolerant plants has shown that the cell wall fractions are a major site of metal localisation. A superior capacity for binding metals in the cell walls of tolerant plants may exert a protective role by preventing the metal reaching more sensitive sites in the roots (143,141).

Zinc tolerance in Agrostis tenuis has been studied with regard to

immobilisation of zinc in cell walls. A close correlation between increased zinc tolerance and increased capacity for binding has been found, the zinc was released by digestion of the cell walls with cellulase (31).

In Agrostis stolonifera, a pectate extract from the cell walls of a zinc-tolerant clone contained five to six times more zinc than a similar extract of cell walls from a non-tolerant clone of the species (143).

Copper in tolerant ecotypes of both A. tenuis and A. stolonifera is also localised in the cell wall (141). Farago, Mullen, Cole and Smith found from chemical fractionation studies of Armeria maritima, that copper was largely stored in the carbohydrate of the cell walls (126).

Although the cell wall may play a role in the metal tolerance of certain species for some metals, it does not fully explain the tolerance observed, nor is it a universal mechanism.

If the carboxyl groups of the cell wall are important in metal binding, then zinc-tolerant clones of A. stolonifera should also be copper tolerant, which they are not, since copper has a greater affinity for carboxyl groups than zinc (143). Furthermore, if zinc tolerant and non-tolerant clones of A. tenuis are grown in zinc amended culture solution both plant types accumulate similar quantities of zinc in their leaves (31,145). The cation-exchange capacity of roots of zinc-tolerant and non-tolerant roots of Deschampsia caespitosa and Anthoxanthum odoratum are similar i.e. acidic polysaccharides do not contribute more to wall structure in zinc tolerant plants as would be expected.

It is possible that the membranes of tolerant genotypes may be structurally different (145). Increasing the concentration of copper was found to cause increasing leakage of K^+ from non-tolerant and zinc tolerant A. tenuis plants, but much less leakage from the copper-tolerant

root tips. These results were interpreted as meaning that zinc ions did not damage the plasmalemma whereas copper ions did, suggesting that plasmalemma modifications are involved in copper-tolerance but not in zinc-tolerance (141).

Enzymic adaptations

A study of the inhibitions by copper of cell wall acid phosphatases of a copper tolerant and a non-tolerant clone of A. tenuis showed that less stable metal complexes were formed with enzymes of the tolerant clone (141,143). Similarly significant differences in the inhibitor constants for Zn-inhibition of acid phosphatases in Anthoxanthum odoratum have been observed (31). However other workers have not detected any differences in the degree of enzyme inhibition shown by non-tolerant and tolerant plants (31,143,141,142).

Efflux

Brookes, Collins and Thurman studied zinc tolerance in non-tolerant and tolerant clones of Deschampsia caespitosa. They reported that both clones were capable of actively pumping zinc across the plasmalemma into the external medium. Only the tolerant clone was capable of actively pumping zinc out of the cytoplasm of root cells into vacuoles when exposed to levels of zinc up to 1 mM, this process was inhibited above 0.1 mM zinc in the non-tolerant clone (142).

Role of organic acids

Mathys has suggested a mechanism of zinc tolerance based upon complexing of metals with various organic compounds including organic acids, thus reducing the activity coefficients of the free ions and their toxicity (146).

Lee, Reeves, Brooks and Jaffré found that most of the nickel in six species of nickel-accumulating plants of New Caledonia was present as a negatively charged citratonickelate (II) complex (147).

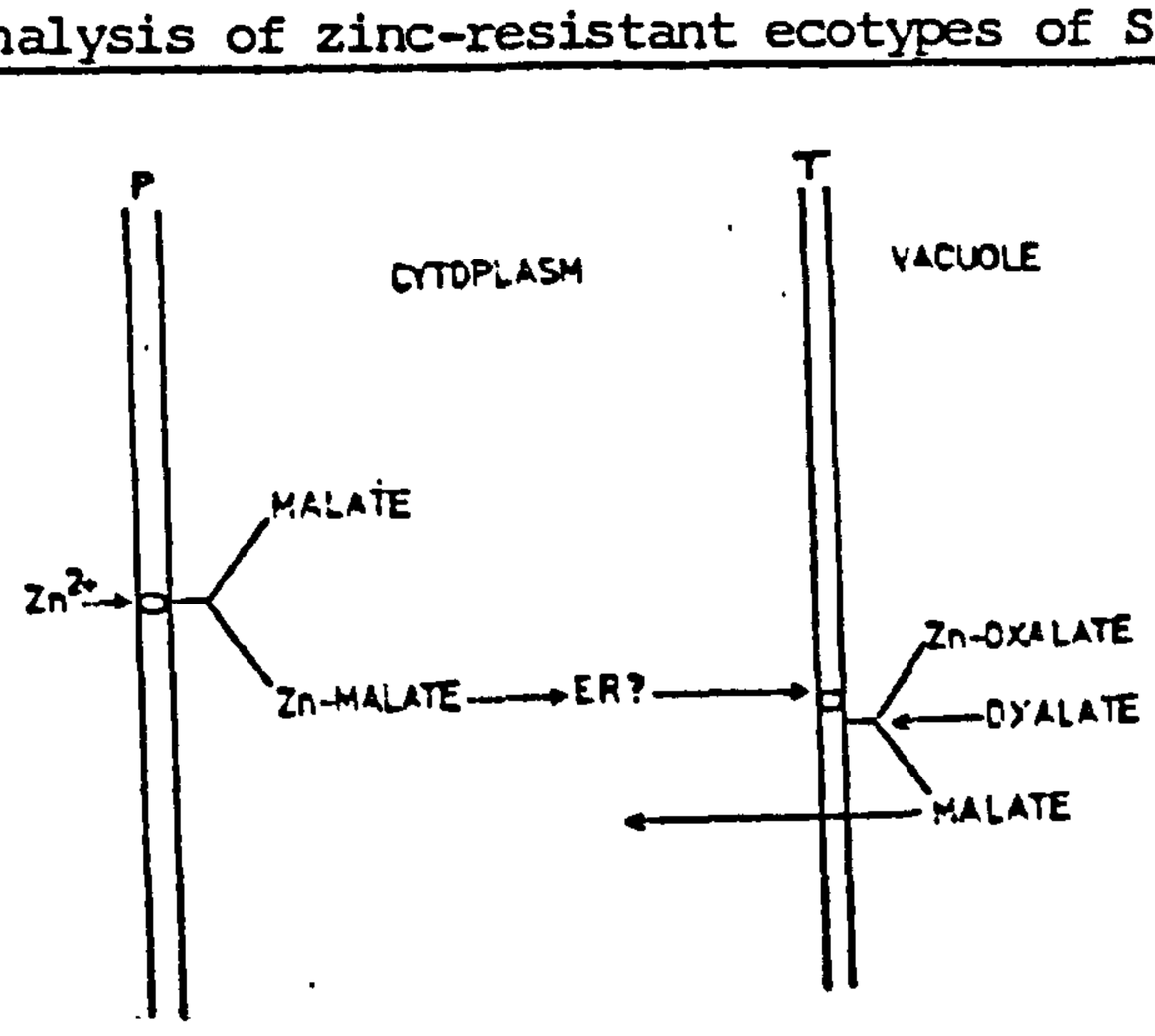
The general occurrence of citrate in plants and its ability to form chelates with most metal ions suggest that this ligand may play a role in transporting metals in the xylem fluid (148).

Other reports have sought to correlate high levels of malic acid in plants to their zinc or zinc-copper tolerance. Such plants also show elevated levels of citrate, but to a lesser degree than malate (143).

Thurman and Rankin investigated the relationship between metal tolerance and accumulation of organic acids using zinc-cadmium tolerant and non-tolerant clones of Deschampsia caespitosa. They found that zinc significantly increased both citrate and malate levels in the roots of the tolerant clone but had no significant effect on the levels of these acids in the roots of the non-tolerant clone. Cobalt, copper, mercury and nickel had no significant effect on the levels of these acids in either clone (149).

Mathys proposed a model, which envisages the malate ion functioning so as to afford a protective passage for zinc en route to the vacuole where other ligands provide more permanent storage (146).

Figure 6 : Model of a mechanism of zinc-tolerance in herbaceous plants, (based on the analysis of zinc-resistant ecotypes of Silene cucubalus)



ER = endoplasmic reticulum

T = tonoplast

P = plasmalemma.

If acids such as citrate and malate are involved in tolerance, it is unlikely that these molecules themselves are responsible for the specificity of tolerance. If the stability constants of citrate chelates are compared, it would be expected that a plant showing copper tolerance would also show zinc tolerance, but it is known that this is not always the case.

Role of proteins

Because metallothionein is known to play an important role in the binding of certain heavy metals in animals, several research groups have considered the possibility that it may play a similar role in plants.

Metallothionein was first isolated from equine renal cortex by Margoshes and Vallee in 1957. The metalloprotein preparation contained 2.0-2.5% cadmium and 0.5-0.6% zinc and was of low molecular weight. Later studies by Kägi and Vallee showed that it contained a high content (4%) of sulphur, mainly as cysteine residues and was found to be deficient in aromatic amino acids. Its name, metallothionein, derives from the sulphur rich protein, thionein. Due to the absence of tyrosine and tryptophan the protein had little or no absorbance at 280 nm, but cadmium-thionein had an absorption maximum at 250 nm, characteristic of cadmium-mercaptides (151,150).

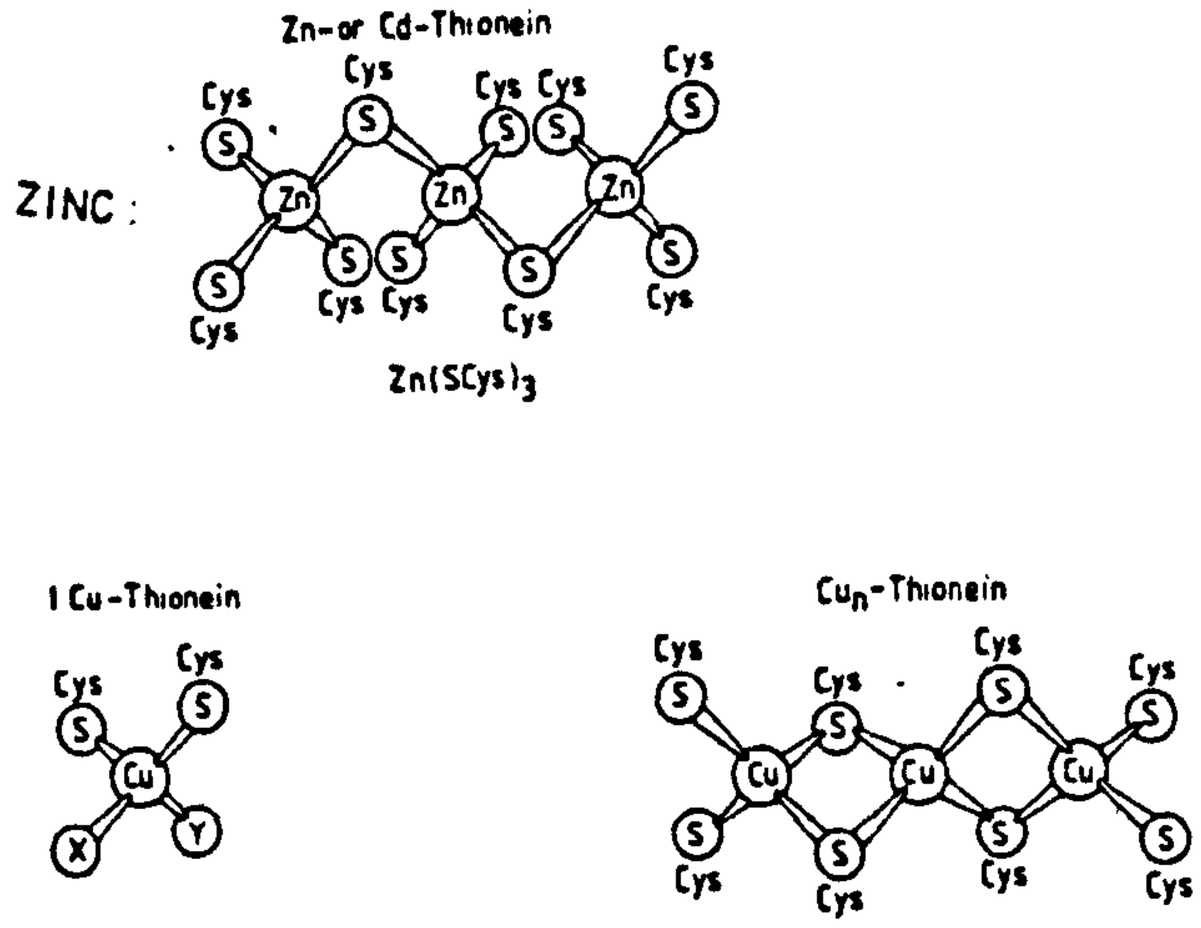
Metallothionein isolated from human kidney contained copper and mercury as well as cadmium and zinc. Silver was found to displace all but mercury from the protein (150).

Synthesis of this protein is inducible and provides a protective mechanism against these heavy metals, but the protein may also have a normal function in the regulation of the metabolism of Cu^{2+} and Zn^{2+} cations (150).

Metallothionein is now used as the generic term, with different

metallothioneins defined by reference to the principal bound cation(s) (e.g. cadmium-thionein, zinc-thionein, (copper,zinc)-thionein)(150). It is suggested that one three-coordinated sulphur is found in the Zn- or Cd-thioneins, whilst two such sulphur species might be assumed to surround the Cu (I) in the Cu-thionein (150).

Figure 7 : Metal-thiolate chromophores in metallothioneins



Metallothioneins are widely distributed (152), having been found in both eukaryotes and prokaryotes (153).

There have been a series of investigations into the possibility of metallothionein-like proteins in plants. Rauser and Curvetto isolated Cu-thionein from the roots of a copper-tolerant clone of Agrostis gigantea (exposed to 16 μM of $CuSO_4$ in the nutrient solution for 1 week). The protein was identified on the basis of thermal stability, low molecular weight, high half-cysteine content, metal binding capacity and ultra violet spectrum (154).

Casterline and Barnett isolated and characterised d-binding components in soybean plants. The plants were exposed to ^{109}Cd. The

^{109}Cd was found to be bound to macromolecules of 1) over 50,000, 2) 13,800 and 3) 2280 molecular weight. The 13,800 molecular weight ^{109}Cd-bound component was found to be inducible by cadmium. It had a high ultra violet absorbance at 254 nm and a low absorbance at 280 nm i.e. characteristics similar to those of mammalian metallothionein. The 2280 and over 50,000 molecular weight fractions were thought to be non-specific binding to normal plant cell components (155).

Bartolf, Brennan and Price exposed tomato plants to a daily application of $CdCl_2$ at 2 μg Cd^{2+}/ml, for at least two weeks. A Cd-binding protein was isolated from the roots, (156), and Girling and Peterson found that cadmium was able to form organometallic compounds with barley roots and shoot proteins (157).

A cadmium-thionein like protein was reported present in cadmium treatedrice plants by Kaneta, Hikichi, Endo and Sugiyama. The protein exhibited metallothionein characteristics but differed from animal metallothionein in molecular weight (estimated to be 33 100) (158).

Yeast copper-thionein (molecular weight 4800) contains four copper and eight cysteine residues. No aromatic residues were detected. Approximately 33% of the total residues are attributable to glutamate and aspartate (159).

Premakumar, Winge, Wiley and Rajagopalan isolated a 'copper-chelatin' from yeast and mung bean exposed to copper (II) ions. The protein was described as being similar to rat liver Cu-chelatin (160). It has since been suggested that this complex was an artifact of isolation (141).

There is no procedure in general use for the direct determination of metallothionein. In most studies, analyses are made after separation of the metallothionein by gel permeation, and thus are preceded by the preparation of a soluble fraction from the tissue. Some loss of

metallothionein may occur in this preparative step - a problem when the metallothionein content is low (150).

In theory it is possible to attain any degree of resolution in gel chromatography by using a sufficiently long chromatographic column. But in practice increasing the length, L, is a relatively inefficient way of improving the separation, because resolution increases with $\sqrt{L}$. Doubling L will thus increase resolution by only 40%, while the flow rate will generally fall by at least 50%. The practical limit of L is reached quickly, columns longer than 100 cm are of little use (161).

An incorrectly packed column, or one allowed to become partly clogged or filled with air, will give poor results. Flow irregularities in the mobile phase will produce excessive peak broadening. Gels that contain particles of widely differing sizes must therefore be avoided (161).

Whilst gel permeation does not yield the pure metallothionein, it is probable that all bivalent heavy metal ions in this fraction are thionein-bound. As Zn^{2+} is bound less firmly by thionein than is Cd^{2+}, Hg^{2+} or Cu^{2+}, native zinc-thionein, even if fully loaded in vivo, may be expected to lose some Zn^{2+} during gel permeation and other purification procedures.

The soluble metallothioneins isolated by gel permeation may be resolved by ion-exchange chromatography on either DEAE-cellulose or DEAE-Sephadex. Elution from the ion-exchange column is carried out using a linear gradient of a suitably buffered solution. Most of these fractionations have used a linear concentration of either Tris-HCl buffer, pH 8.6 or NaCl in a dilute Tris-HCl buffer. Since high concentrations of NaCl cause intense light scattering and thus interfere in AAS measurement, the eluted sample must first be desalted (150).

Preliminary fractionation steps that employ ammonium sulphate, organic solvents and rivanol should be avoided if the native state of

the protein is to be preserved. The alternative is the storage of large volumes of dilute solutions of metallothioneins with the inherent risk of denaturation. Oxidation of the metallothioneins may be a problem. It has been suggested that the presence of two or more metallothionein components resolved during ion-exchange is due to alterations of the metallothioneins during the isolation procedures (150).

The importance of the metallothionein-like proteins is yet to be determined. They may be involved in metal sequestering only or they may play a role in the transport of metals to a storage site (150,153).

SECTION 2 : METHODS OF ANALYSIS

I. Spectroscopic Methods

a) Atomic Absorption Spectrometry (AAS)

b) Atomic Emission Spectrometry (AES)

c) Spark-Source Mass Spectrometry

d) Neutron Activation Analysis (NAA)

II. Electroanalytical Techniques

a) Polarography

b) Anodic Stripping Voltammetry (ASV)

III. Gel Chromatography

IV. Ion Exchange Chromatography

V. Speciation Schemes

SECTION 2 : METHODS OF ANALYSIS

Due to our improved knowledge of the deleterious effects caused by very small quantities of heavy metals, it is necessary to determine even smaller concentrations with higher accuracy and precision (162).

A comparison of some analytical techniques used to measure the four trace metals of interest is given below :-

Table 17 : Comparison of detection limits of some methods for ultra-trace analysis

Method	Detection limits Cd	Cu	Pb	Zn	Concentration units	Ref.
NAA	1-10	0.1-1	100-1000	10-100	ng	163
Spark Source Mass Spec.	0.03	0.08	0.3	0.1	ng	163
FAAS	0.7	2.0	0.5	0.008	ng/ml	164
ETAAS	0.01	0.3	0.5	0.008	ng/ml	164
AES	0.8	0.01	0.1	50.0	µg/ml	165
ICPES	2	1	2	2	ng/ml	165
DPASV	0.005	0.005	0.01	0.04	ng/ml	164
NPP	0.1	0.06	0.2	0.07	µg/ml	166

1) Spectroscopic Methods

a) Atomic Absorption Spectrometry (AAS)

The first paper demonstrating the use of AAS as an analytical tool was published in 1955 by Walsh (167). Standard commercial equipment became available about 1960 (168).

When a solution containing a metallic species is atomised in the flame, the majority of the metal atoms remain in the non-emitting ground

state. If irradiated with light of their own characteristic resonance wavelength, these ground-state atoms will absorb some of the radiation, the absorbance being proportional to population density of atoms in the flame. Atomic absorption spectrometry is based upon this principle (168).

The most commonly used atomiser is the chemical flame, based upon the combination of a fuel gas (e.g. acetylene) with an oxidant (e.g. air or nitrous oxide). The sample solution is aspirated directly into the flame, via a nebuliser in which the passage of the oxidant creates a partial vacuum by the Venturi effect. The choice of oxidant depends upon the flame temperature and composition required for the production of free atoms (169).

One of the most attractive aspects of liquid sample introduction is its relative simplicity and reliability. This, allied with the fact that a sample dissolution step is often necessary to provide suitable sampling statistics, is probably the reason for the overwhelming use of liquid sample introduction to all branches of atomic spectroscopy. However, the pneumatic nebulisers used for liquid sample introduction are extremely wasteful. In AAS, nō higher than 10% efficiency can be expected; furthermore a 60 s interval is necessary between introduction of successive samples to the spectrometer (170).

The most direct approach to overcoming poor sample transport efficiency is to allow more of the pneumatically generated aerosol to pass directly into the flame (or plasma). Some early atomic absorption spectrometers used a total consumption burner placed directly beneath the laminar flame slot as a means of high-efficiency sample introduction. Such instruments were found to suffer from poor precision and high vaporisation interferences and therefore this method of introduction has largely been abandoned.

In FAAS, any improvement in transport efficiency must not be achieved

at the expense of introducing large aerosol drops to the flame :-

$$d_x^3 = d_o^3 \left(\frac{C}{\rho}\right) 10^6$$

where C is the solution concentration (μg/ml),

d_o is the initial diameter of the drop (μm),

ρ is the density (g/cm^3) of the dry salt crystal, formed after solvent loss

d_x is the diameter of the dry salt crystal (μm).

From the equation, it can be seen that the larger the drop diameter, the larger is the crystal diameter and hence, the greater is the interference (170).

When non-volatile matrices are present in high concentration, the vaporisation rate of the analyte will be inhibited. In lower temperature flames, such as air-acetylene, this may lead to severe vaporisation interferences (170).

Aerosols produced from atomic absorption and ICP nebulisers have both extremely wide drop size ranges, and very turbulent gas flow patterns.

The aerosol modifying processes that converts the primary aerosol (i.e. the aerosol emerging from the nebuliser) to the tertiary aerosol (i.e. the aerosol arriving at the atomiser) are caused by devices such as spray chambers, impact beads and impaction surfaces, which are placed in the aerosol path. Aerosol modifiers typically remove large drops from the stream, and allow only drops of less than a certain size (~ 7.5 μm) to pass, so resulting in the production of the secondary aerosol. The transition from primary to tertiary aerosol is accompanied by a large reduction in mean drop size of the aerosol. The mass fraction of aerosol contained in the larger drops is substantial and accounts for the high rate of wastage found with pneumatic nebulisation, as essentially

all the large drops pass to waste (170).

The ground-state metal atoms produced in the flame are subjected to radiation from a hollow cathode lamp, consisting of a glass envelope containing a cathode and an anode (169), and filled with an inert gas (167). The cathode is made of the chemical element for which analysis is of interest (169).

The monochromator separates, isolates and controls the intensity of radiant energy reaching the detector (169), while the photomultiplier converts the radiant energy from the source into an electrical signal for amplification and measurement by the readout system (169,171).

Interferences encountered in AAS can be classified into the following categories :-

i) Spectral

ii) Flame emission

iii) Chemical

iv) Matrix

v) Non-specific scatter

vi) Ionisation.

The majority of difficulties arise from categories (iii) to (vi).

i) Spectral interference (172)

Spectral interferences in the past were experienced typically if, in a given solution, element A was being determined in the presence of element B. If the source contained both elements and the absorption lines of these could not be resolved by the monochromator, element B would cause an interference. Most of the interferences have now disappeared due to improvements in the purification techniques of the cathode and to some extent in monochromator quality.

ii) Emission interference (172,171)

Such interference is caused by emission of the element at the same wavelength as that at which absorption was occurring. All modern instruments use a.c. systems which are 'blind' to the continuous emission from the flame. However, if the intensity of the emission is high, the 'noise' associated with the determination will increase, since the noise of a photomultiplier detector varies with the square root of the radiation falling upon it.

The effect can be reduced by either increasing the source current or by closing down the monochromator exit slit, both methods resulting in an increase in the signal-to-noise ratio.

iii) Chemical interferences (168,172)

A chemical interference can be defined as anything that prevents or supresses the formation of ground state atoms in the flame. It is the most frequently encountered interference. For example, the effect produced by aluminium, silicon, and phosphorus on the determination of magnesium, calcium, strontium, barium and many other metals, being due to the formation of aluminates, silicates and phosphates. In many cases, the moieties are refractory in the analytical flame being used. The interference can be overcome by addition of a chelate, for example, EDTA, which will complex the cation, preventing its association with an anion that could lead to the formation of a refractory compound. Another possibility is the addition of a reagent that will preferentially form a compound with the interfering anion, e.g. the addition of lanthanum chloride to solutions of calcium containing the phosphate anion. The calcium is 'released' due to the preferential formation of lanthanum phosphate.

Alternatively, virtually all chemical interferences may be overcome by using the high-temperature nitrous oxide - acetylene flame.

iv) Matrix effects

Under this heading the interferences encountered include :-

a) Enhancement of sensitivity due to the presence of an organic solvent in the aqueous solution.

b) Depression of sensitivity due to the sample having a greater viscosity than the standard solution.

c) Depression of the result due to a high salt content.

These interferences can be overcome by using one of the following techniques :-

a) The method of standard additions

b) Matching the matrix of the standards with that of the sample

c) Solvent extraction to remove the cation to be determined from the interfering matrix.

v) Non-specific interferences (172,168)

a) Light scattering

The enhancement of an analytical result at the µg/g or sub-µg/g levels due to the solution containing a high concentration of dissolved salts. The effect is due to the presence of dried and semi-dried salt particles in the flame which scatter and absorb the incident radiation from the source. Since the intensity of the transmitted radiation will be decreased, there will be an increase in the absorption signal.

Scattering hasits origin in the induced secondary emission of particles (whether single atoms or molecular aggregates) that lie in the path of radiation. Secondary radiation is scattered only if the particles:-

1) have dimensions approximately the order of magnitude or smaller than the incident wavelengths. Larger particles reflect the radiation. In the UV and visible regions of the spectrum, the scattering particles are those of colloidal size i.e. from 1 nm to 1 µm in greatest dimension.

The scattering of suspended particles is often termed the Tyndall effect. At a given wavelength, the scattering pattern in space is symmetrical for suspended particles smaller than $^1/10$ to $^1/20\lambda$ and is termed Rayleigh scattering (173).

2) Are randomly distributed in a medium of refractive index different from their own.

The intensity of Rayleigh scattering, I_s, is given by the following expression :-

$$I_s = \frac{8\pi^4\alpha^2}{\lambda^4 r^2} (1 + \cos^2\theta) I_o$$

where α is the polarisability of the particle,

λ is the vacuum wavelength,

I_o is the incident intensity,

θ is the angle between the incident and the scattered ray,

r is the distance from the centre of the scattering to the detector.

It may also be shown that the polarisability varies roughly as the volume of a particle. Hence the above equation predicts that the scattering will increase strongly as the particles become larger. The equation also indicates the inverse dependence of intensity on the fourth power of the wavelength. The intensity increases very strongly at shorter wavelengths and is most significant below 250 nm (173).

b) Molecular absorption

Although atomic absorption lines of each element are narrow and are easily distinguished from each other, molecular absorption is broadband and may take place over a wide range of wavelengths, frequently including the wavelengths of atomic absorption lines. Broadband absorption may be

caused by unburned fuel in flames or unburned solvent, or fragments of molecules introduced from the sample. Consequently broadband absorption is common in flames.

The effect produced by non-specific interferences can be overcome by one of the following techniques :-

a) solvent extraction to remove the element from the interfering matrix.

b) repeating the determination at a nearby non-absorbing line and subtracting it from the signal obtained at the absorbing line.

c) by using a deuterium background corrector (167). The radiation from a deuterium lamp is measured at the same normal wavelength as the resonance line. The radiation from this lamp fills the spectral slit and therefore a waveband of approximately 0.1 nm or greater will reach the detector. Absorption by molecules in the flame across the entire spectral width, provids a measurement of the molecular background absorption. Atomic absorption also takes place at this wavelength. However, the lines are very narrow (10^{-3} nm) and the total amount of energy absorbed is very small compared with the molecular energy absorbed. If an atomic line is completely absorbed by a waveband of 0.1 nm the total absorption is only 1% which is a negligible amount and may be ignored. Consequently the absorption of the deuterium lamp is a measure of the background absorption.

d) Background correction using the Zeeman effect (174,175)

Continuum source background correctors fail if background absorption is excessive or if the background is structured within the bandpass of the instrument. In these cases the Zeeman effect can be used to obtain a superior correction.

In the Zeeman effect the energy levels of a molecule are split under the influence of a varying strong magnetic field. If the atoms are not

in a changing magnetic field they will absorb at a single wavelength (e.g. the resonance line). A strong magnetic field may then be switched on and the absorption line is split into fine structure with wavelengths greater than and less than the resonance line. If the magnetic field is sufficiently strong the resonance line is eliminated entirely. Either the hollow cathode or the atomiser can be operated in the magnetic field.

Advantages of the Zeeman background corrector are :-

1) a single standard light source is used, thereby eliminating misalignment problems and extra source noise;
2) background correction is at the precise wavelength of the line. Background structure is usually not a problem;
3) background absorbances as high as 2 can be tolerated in some systems;
4) atoms outside the magnetic field give no response;
5) spatial information can be obtained using a restricted magnetic field.

Disadvantages of the system include :-

1) added cost and complexity;
2) reduced sensitivity for many transitions, and shortened linear range;
3) systematic errors can be encountered due to Zeeman shifts in the rotational spectra of diatomic molecules (175);
4) currently Zeeman systems are available from only two manufacturers.

e) Smith-Hieftje background correction (174)

The method applies high current pulses to the hollow cathode lamp. At high lamp current the resonance lines become broadened and self-reversed.

The lamp is operated initially at normal current for measurement of atomic plus background absorption. Then a brief high current pulse (typically 100 mA for 30 μs) is applied to create a broadened, reversed line. By so doing the atomic absorption is greatly reduced, since the

source profile is wider than the absorption line. The line broadening however has little effect on the background absorption, and the difference between low and high current absorbances provides the background-corrected data.

Potential advantages of this method include :-

1) Simplicity
2) No source alignment problems
3) Background correction at a wavelength very close to the absorption line
4) The method is applicable to any atomiser.

Disadvantages include :-

1) Possibility of reduced lamp life, unless applied to modified lamps
2) Reduced sensitivity
3) Not available commercially.

vi) Ionisation interferences (172)

Many determinations require the use of the nitrous oxide-acetylene flame and it is usually under these conditions that ionisation interferences occur. They arise from the energetic nature of the flame which gives ground-state atoms but also excites some atoms to such an extent that one or more electrons are lost and ionisation occurs. The effect will obviously be greatest with elements having low ionisation potentials such as the alkali and alkaline earth metals, e.g. barium is approximately 80% ionised in the nitrous oxide flame. The ground state, therefore, becomes depopulated and the sensitivity will decrease. A similar but opposite effect arises when an easily ionised element is being determined in the presence of another. There will be an enhancement of sensitivity compared with pure aqueous standards (172).

Electrothermal atomisation (ETAAS)

The development of electrothermal atomisers can be traced back to the work of A.S. King in 1905 and 1908. His aim was to obtain emission spectra, as nearly as possible, solely by the effect of heat. Then in 1959 L'vov reported the application of electrothermal furnace atomisers for quantitative atomic absorption analyses (176).

Various electrically heated furnaces have been described in recent years; a fixed sample volume is introduced into the furnace and after thermal pretreatment is rapidly atomised. A transient signal is produced, whose height or area is proportional to the quantity of element under study. In a graphite furnace atom cell a substantially higher peak concentration of atoms may be expected compared with a flame cell. This gain results directly from avoidance of the dilution and expansion effects that occur in flame cells. To assist the formation and maintenance of a dense free atom fraction of the element for atomic absorption analysis it is also an advantage that the chemical environment can be controlled by the use of an inert gas atmosphere, usually argon (169).

Many commercial electrothermal atomiser designs are available; including versions of the carbon furnace, cup, and rod, and the tantalum filament (166). The electrothermal atomiser replaces the burner in the spectrometer, where it performs the same function as the flame, but from 100 to 1000 times more efficiently in terms of sensitivity. A comparison of graphite furnace and flame AAS sensitivity is given in Table 18 for Cd, Cu, Pb and Zn (176).

Table 18 : Sensitivity of graphite furnace and flame AAS for Cd, Cu, Pb and Zn (in pg) in aqueous solutions (Flame data are based on a sample volume of 1 ml)

Element	Graphite furnace	Flame
Cd	1	30 000
Cu	30	100 000
Pb	20	500 000
Zn	1	20 000

The furnace power supply and controller enables the basic steps normally considered to occur in the flame method of atomisation to be carried out in a sequential form, each stage governed by the particular phase of the controller programme. In its simplest form the programme will consist of three readily identifiable stages, namely dry - ash - atomise. Each of these stages has to be carefully optimised to obtain the best results for any particular analysis (172). Typical values are solvent evaporation 120^{o}C, dry ash 400^{o}C and atomisation < 3000^{o}C (179).

Furnace atomic absorption has certain disadvantages. The flame is more precise, faster and requires less skill. Moreover, contamination can be a problem at these ultra-trace levels (169). The carbon tube or device must be replaced due to oxidation problems as a result of the high temperatures employed, and it is necessary to use the method of standard additions to quantitate each sample (177).

The advantages and disadvantages of AAS are summarised below :-

Advantages :-

More than 70 elements can be determined at low levels and at moderate cost since commercial instrumentation is readily available. It is a rapid versatile technique for the determination of metals in water and since water samples can be introduced directly into the spectrometer, often only a minimum of pretreatment is necessary. Single or multi-element analysis can be automated or computer controlled, giving good selectivity. Solid sampling may be possible. Interferences are few and well documented and therefore AAS is ideally applicable to the analysis of the wide range of samples encountered (164,178,179). The reproducibility of analytical absorption signals in flame AAS for repetitive introduction of liquid samples can be better than 1% i.e. a relative standard deviation of 0.01. For ETAAS this is 3%, i.e. a relative standard deviation of 0.03, with automated sampling systems the relative standard deviation values can be as low as 0.05. For trace element determinations the precision is good with RSD values of 0.1 (180).

Disadvantages :-

The method is destructive, and suffers from matrix or interelement interferences. Solid sampling can result in poor precision (164,178). Reliability - insensitivity of the reading to alteration of instrumental parameters - is sometimes limited (177).

b) Atomic emission spectrometry (AES)

Atoms are excited when energy is absorbed and can emit radiant energy when returning to the ground state. The photons of energy emitted are characteristic of the excited atoms in the system, and forms the basis of the technique of AES (172).

The number of atoms (N_1) that are in an excited state compared to the number (N_o) in the ground state (for a system in effective thermal equilibrium) is given by the Boltzman equation :

$$N_1 = N_o \frac{g_1}{g_o} \exp\left(\frac{-E}{kT}\right)$$

where E is the energy of the excited state above the ground state (J)

g_1, g_o are the statistical weights of the excited and ground states respectively

k is the Boltzman constant (J/K)

T is the absolute temperature.

From the equation it can be seen that the ratio of N_1/N_o rapidly decreases with decreasing wavelength (increasing E). Except for elements with long-wavelength resonance lines (e.g. potassium) at extremely high temperatures, the number of atoms in the lowest excited state is negligible in comparison to the number in the ground state (168).

A flame emission spectrometer consists of an atom source, a monochromator and detector. The technique does not require a hollow cathode lamp (172).

The flame background signal is very dependent on the solution uptake rate of the nebuliser. The greater the solution uptake rate the lower the flame background signal. This effect is caused by cooling of the flame by the nebulised solvent.

Spectral interference is the most serious form of interference, but any extraneous substance that causes a variation in the number of ground state atoms in the flame will interfere (168).

The advantages and disadvantages of AES are summarised below :-

Advantages :-

More than 40 elements can be determined, and most atomic absorption spectrophotometers can be used in flame emission studies. The method does not require a hollow-cathode lamp for each element to be determined and hence misalignment problems are avoided. It is possible to perform rapid qualitative flame emission analysis by the wavelength scanning technique. Calibration curves can often be used over wider concentration ranges in flame emission than atomic absorption. With simple instrumentation it is possible to obtain very good precision when using signal integration with the flame emission technique. In general, at concentration levels above 100 to 200 times the detection limit in simple matrices, the precision should be 0.3 to 1% RSD, when signal integration techniques are used. Wavelength scanning techniques can give precision of 1 to 5% (168).

Disadvantages :-

Any slight temperature fluctuation in an atomic vapour will have a pronounced effect on the excited state atom population utilised in atomic emission. Spectral interference is often observed in flame emission owing to the relatively large number of atomic lines, and broad

molecular bands that can be observed in emission. The flame emission techniques are not very sensitive for elements whose main resonance lines lie below 270 nm (e.g. Cd, Zn), as the flame temperatures are too low to excite sufficient atoms to the first excited state. Similarly, certain elements are not appreciably broken down to atoms even by a fuel-rich nitrous oxide - acetylene flame (e.g. U, La). More operator skill is required in the interpretation of flame emission results (168).

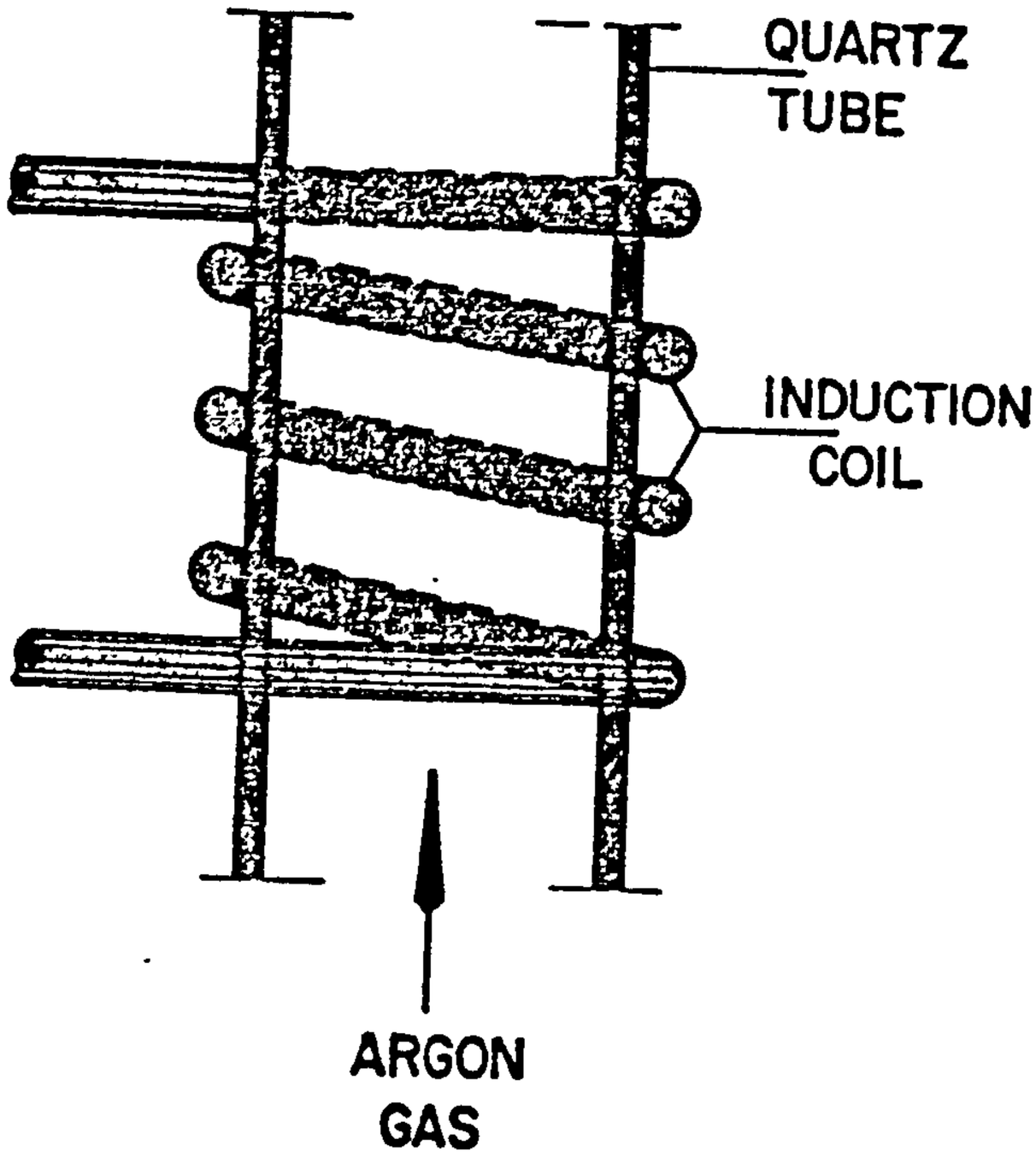
Inductively coupled plasma emission spectroscopy

In spite of the success achieved by AAS and AES, during the last decade, many analysts have realised that flames, which involve violent chemical reactions are not ideally suited for the production of free atoms which has led to the investigation of various electrically generated "flames" or plasmas possessing higher gas temperature and less active chemical environments. Another motivation for exploring alternative means of generating free atoms and releasing them into a reservoir suitable for spectroscopic observation was the increasing necessity of performing simultaneous multielement determinations at the nanogram/millilitre level for many trace constituents, often on large numbers of samples, and occasionally under conditions for which sample volume was limited (165).

An inductively coupled plasma model is given in Figure 8. The quartz tube of approximately 2.5 cm diameter is placed inside a coil connected to a high frequency generator operating typically in the 40 to 50 MHz range at generator output levels of 2 to 5 kW.

To form a plasma it is necessary to "plant" a seed of electrons in the coil space; the simplest way to do this is to "tickle" the tube with a Tesla coil. The high-frequency currents flowing in the induction coil generate oscillating magnetic fields whose lines of force are axially oriented inside the quartz tube and follow elliptical closed paths outside the coil. The induced axial magnetic fields, in turn, induce

Figure 8 : Inductively coupled plasma model



the seed of electrons and ions to flow in closed annular paths inside the quartz tube space, the electrons being accelerated on each half cycle. The accelerated electrons (and ions) meet resistance to their flow, heating is a natural consequence and additional ionisation occurs. These steps lead to the almost instantaneous formation of a plasma (i.e. a gas in which the numbers of positive and negative ions are approximately equal).

The plasma formed in this way attains a gas temperature in the 9000 to 10,000 ^{o}K range in the region of maximum eddy current flow. Thermal isolation of the plasma, to prevent overheating of the quartz containment cylinder, is achieved by introducing a tangential flow of argon. The flow cools the inside of the outermost quartz tube and centres the

plasma radially in the tube. There is another lower velocity flow of argon which transports the sample to the plasma, either as an aerosol, a powder, or a thermally generated vapour.

If the plasma is generated at lower frequencies ($\sim$5 MHz), a teardrop-shaped plasma is formed. Sample material that approaches the plasma follows a path around the outside surface. An increase in the oscillating frequency of the power source causes the eddy current to flow more closely to the outer or skin portions of the plasma, and an annular shaped plasma is developed. The gas temperature in the axial channel of the eddy current flow regions is $\sim$ 7000 ^{o}K. This temperature is a factor of two higher than those achieved in the hottest commonly used combustion flames. Typical residence times of the sample in the plasma before the observation height is reached is 2.5 ms. The combination of high temperatures and relatively long particle-plasma interaction times should lead to complete solute vaporisation and a high, if not total, degree of atomisation of the analyte species in the core of the plasma. Once the free atoms are formed, they occur in a chemically inert environment, as opposed to the violently reactive surroundings in combustion flames. Thus, their lifetime, on average, should be longer than in flames (181).

Traditionally, samples have been introduced into the plasma by pneumatic nebulisation, but because of the problems associated with nebulisation outlined earlier, discrete sample introduction using both the volatile hydride technique and electrothermal atomisation technique have been applied (180).

The advantages and disadvantages of ICPES are outlined below :-

Advantages :-

Simultaneous multi-element determination of metals and metalloids over a wide concentration range in solutions is possible. The ICP source can allow detection limits in the ng/ml level for many metals, with linear dynamic concentration ranges typically 5 orders of magnitude and freedom from chemical, condensed phase and vapour phase interferences (180). Determination at the ultra-trace level, on µl or µg samples, is possible. Modern ICP instruments allow a high rate of sample throughput with a minimum operator attention. The flexibility of utilising many emission lines for each element allows different elements in a sample at various concentrations to be determined, at a single dilution factor, and hence less labour and less chance of dilution errors or contamination (182).

Disadvantages :-

The ICP is expensive (both to purchase and operate) and is therefore not readily available (183). The all-glass concentric nebulisers often used with ICP are delicate and particle blockage can be an irreversible process (184). Detection limits for Cd, Cu, Pb and Zn may be superior with other techniques (Table 17). The sensitivity is limited to approximately 0.1 to 1 µg/ml for most elements. The limited sample size may be a handicap, since the sample is consumed in this process and is not available for additional analysis (185). High solvent loading may extinguish the plasma (170).

c) Spark-source mass spectrometry

Spark-source mass spectrometry is a means of obtaining mass spectra of involatile, inorganic compounds. The powdered sample is added to a relatively large amount of graphite, the resultant mixture is compressed into a rod. Two rods are mounted close together as electrodes in the ion source of a mass spectrometer and an electrical potential (> 10 kV) is applied between them. An electric discharge (spark) occurs in the gap between the rods (electrodes) and ions of the electrode material are formed. The ions are accelerated out of the ionisation region, analysed in the mass spectrometer and detected on a photographic plate. The method is used because all ions are detected simultaneously, thereby compensating for erratic formation of ions (186) by fluctuations in the ion beam.

Spark-source mass spectrometry has little application in organic chemistry. Covalent bonds are easily broken under the conditions of ionisation, resulting in too large a number of fragment ions. Its greatest potential lies in the elemental analysis of involatile inorganic substances. A large number of elements (X) can be detected simultaneously, as X_a^{b+} where a and b are small integers. The measurements are quantitative since the abundances of ions are related to the degree of darkening produced on the photographic plate (186).

Spark-source mass spectrometry has been used to determine 37 elements detected at levels of 0.1 µg/g or higher in dental tissues (187). The use of the method has been reported for the estimation of some 25 elements in hair, with a sensitivity of 0.1 µg/g (187). The method has been applied to the determination of 35 element impurities in rocks. Precisions of less than 5% were maintained on impurity concentrations in the range 7 µg/g to 200 µg/g (188). Spark-source mass spectrometry of 25 metals in the ash of various plants has also been reported, with a sensitivity of 10 ng/g and an accuracy and reproducibility of $\pm$ 10 to 25% (187). The technique is also applicable to tissues such as kidney tumour and bone, as well as archaeological samples and the determination of traces of impurities in semiconductors (186,187).

A summary of the advantages and disadvantages of this technique is given below :-

Advantages :-

The technique allows overall elemental coverage with low detection limits and a favourable speed of analysis, coupled with good selectivity (163,178).

Disadvantages :-

The technique is destructive, difficult to automate and is not readily available due to its high cost (163,178). Sensitivity can be limited due to gas scattering or background carbon levels (189).

Inductively coupled/Mass spectrometry (ICP/MS)

In ICP/MS, the sample is rapidly dissociated and ionised in the plasma. The resulting ions are then admitted through an aperture into the vacuum system of a quadrupole mass spectrometer, where the ICP as an ion source makes it possible to use the whole range of conventional ICP sample introduction methods, including nebulisation, hydride generation, and electrothermal vaporisation.

The advantages and disadvantages of the technique are outlined below :-

Advantages :-

The detection limits in ICP/MS are in general better than in plasma emission spectrometry, and the ICP/MS makes it possible to determine elemental isotopic ratios and to use isotope dilution for quantitation.

Disadvantages :-

Due to its very high cost, the system is not readily available.(190), and very few instruments have been delivered.

d) Neutron activation analysis (NAA)

In neutron activation analysis, the sample to be analysed is bombarded with slow (thermal) neutrons, some of which are captured by the atomic nuclei to produce radioactive isotopes. Because these artificially activated nuclei emit characteristic radiation (just as the naturally radioactive elements do), they can be determined (191).

A summary of the advantages and disadvantages of NAA is given below :-

Advantages :-

The technique provides overall elemental coverage at low detection limits, if both fast and slow neutron sources are available. NAA is non-destructive, and has good selectivity, precision and accuracy, being able to detect as little as 10^{-15} g of an element (163,178).

Disadvantages :-

NAA is very expensive and access to a nuclear reactor is required, so that it is restricted in ready application. The determination of lead is not possible (164,178,191).

II. Electroanalytical techniques

a) Polarography

The polarographic method of analysis is based on the observations of Heyrovsky in 1922, regarding the current-voltage relationships obtained on electrolysing solutions using mercury electrodes (192,193).

The working electrode consists of easily polarised droplets of mercury emerging regularly (at a rate of one drop every 3 to 5 seconds) from the end of a fine bore (0.05 to 0.08 mm i.d.) capillary tube. This, then, is the dropping mercury electrode (DME) of classical polarography (192,914).

One key characteristic common to all modern polarographic instrumentation is "potentiostatic" control of the working electrode potential. The potentiostat controls the potential at the working electrode-solution interface, eliminating errors owing to solution resistance and modern instruments are therefore usable in a wide range of systems (195).

A potentiostat accomplishes this end by making use of a three electrode system. Here, a reference electrode of constant potential, inserted in the system and positioned as closely as possible to the working electrode, is connected to the instrument through a circuit which draws essentially no current from it. There is thus no effective current flow between the tip of the reference electrode (or its connecting bridge) and the instrument and thus no voltage drop. The electrical output of the circuitry within the instrument is then the voltage right at the tip of the reference electrode. Sufficient compensating potential is applied to the counter electrode to insure that the potential at the reference electrode tip is that desired, even if solution resistance is sufficiently high as to cause appreciable voltage drop when currents flow through the solution (195).

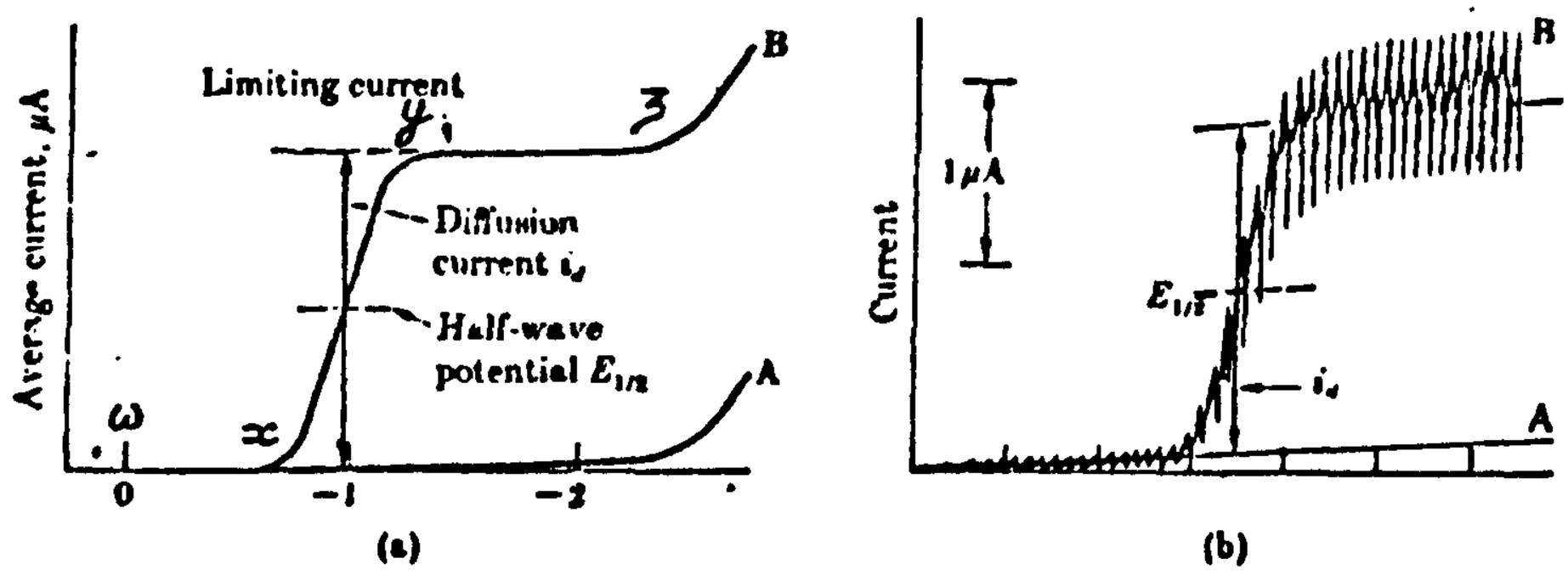
Typically a linearly increasing voltage (in a negative direction) is applied to the DME with respect to the reference electrode (SCE or standard Ag/AgCl electrode) in a cell (192). The mercury drop is allowed to increase in size until there is a minimum change in area (usually after the first 1.5 to 2 seconds of drop life). Then the current in the cell is sampled just prior to the dislodgement of the drop. The sampled current is stored in a memory and read out to the recorder until the next measurement is taken. In this way the recorder plots a curve representing the peak current flowing during each drop's life cycle, and is devoid of drop-induced fluctuations (194).

The current is produced by the reduction of ions at the DME (or oxidation if the electrode is operated anodically). Since the solution is not stirred, the current is due to :-

1) the migration of ions to the working electrode in the electrical gradient set up, and
2) the diffusion of ions in the concentration gradient produced by the removal of ions from solution in the proximity of the working electrode (192).

Only the latter current is required, since its magnitude is dependent on the concentration of the reducible substance. The effect of the migration is usually eliminated by adding an excess of an inert "supporting electrolyte". The ions of this electrolyte quickly migrate to relieve the electric fields but do not undergo any electrochemical reaction at the electrode (194). Thus the current observed is limited essentially by the process of diffusion (192). The important features of a dc polarogram are shown in Figure 9 (196).

Figure 9 : Potential of DME vs. SCE in volts (196)



(a) Smoothed current-voltage curves for dc polarography

Curve A = deoxygenated supporting electrolyte alone;

Curve B = single electroactive species and supporting electrolyte.

$E_{\frac{1}{2}}$ = half wave potential

i_d = diffusion current.

The half-wave potential ($E_{\frac{1}{2}}$) is characteristic of the reducible cation and hence provides qualitative information about the cation (194).

The magnitude of the diffusion current i_d provides quantitative information about the cation (194), since, from the Ilkovic equation (page 81) it is directly proportional to concentration.

The current oscillations in Figure 9(b) are caused by the growth and detachment of the mercury drop (166).

The curve falls into three separate portions (192,196) :-

i) From zero applied potential (at w) to the decomposition potential (at x) - only a very small current, i.e. the residual current passes through the cell (represented by wx, this is due to impurities in the solution).

ii) At x an appreciable rate of discharge of the sample ions at the DME is attained resulting in an increase in current flowing through the

the solution. The curve at x suddenly turns upwards and the current increases rapidly for small increases in the applied voltage until y is reached when the current becomes constant again.

iii) After y the current no longer increases with the applied voltage and the steady current represented by yz is referred to as the 'limiting' or 'diffusion' current.

After z there is a large diffusion current that flows when the voltage becomes sufficiently negative to reduce either the solvent or the cation of the "inert" electrolyte (192).

Even when no reducible species is present in solution an appreciable current must flow to charge the double layer capacitance at the surface of each growing drop up to the new applied potential. At the solution-electrode interface, a separation of charge takes place which makes the interface appear as a large capacitor to the external circuitry. Current is required to charge this capacitor, in addition to the current required by any reacting species. Because the electrode surface repeatedly grows to a maximum, then suddenly falls to zero as the drop detaches, the current flowing in the system fluctuates in the same fashion. The charging current appears as a surge at the beginning of each drop when a new capacitor must be charged. The magnitude of this surge increases with applied potential, because the capacitor must be charged to a higher potential, thereby producing a highly sloping baseline. The principal factor limiting the sensitivity of polarography and its accuracy at low concentrations is the charging current. At concentrations of electro-active species of 10^{-3} M or greater, the charging current is negligible compared to the Faradaic current (resulting from the transfer of electrons across the electrode-solution interface (196)) and may be ignored. At concentrations of 10^{-4} M the charging current is an appreciable fraction of the total current and a correction must be made

for it. At concentrations around 10^{-5} M, the charging current is usually larger than the Faradaic current and the precision of the polarographic determination depends principally on how precisely the contribution of the charging current can be estimated, compensated or eliminated at the potential at which the diffusion current is measured (194).

The Ilkovic equation relates the faradaic diffusion current at a DME to the parameters of the DME and the properties of the electroactive species in solution. The Ilkovic equation is :-

$$i_d = 607\, n\, D^{\frac{1}{2}}\, C\, m^{\frac{2}{3}}\, t^{\frac{1}{6}}$$

where i_d is the average diffusion current (μA)

n is the number of moles of electrons involved in the reaction equation

D is the diffusion coefficient of the reducible ion in solution (cm^2/s)

C is the concentration of the reactant (mM/l)

m is the mass of mercury flowing (mg/s)

t is the drop time (s)

Thus the diffusion current is directly proportional to concentration (197).

A more dimensionally correct modification of the diffusion equation, which recognises that the diffusion is toward a curved surface, is :-

$$i_d = 607\, n\, C\, D^{\frac{1}{2}}\, m^{\frac{2}{3}}\, t^{\frac{1}{6}} \left(1 + 34\, \frac{D^{\frac{1}{2}}\, t^{\frac{1}{6}}}{m^{\frac{1}{3}}}\right)$$

The above equations should be used whenever maximum precision is desired for comparing results from different capillaries and for calculating the diffusion coefficient. For analytical applications of polarography, the

original Ilkovic equation is adequate and much more convenient; its errors tend to cancel out in practical use (194).

Polarography is a fairly sensitive technique (limit around 10^{-5} M (198)) for detecting and determining electroactive substances. Dissolved oxygen is electroactive and complications in polarograms are introduced both by the voltammetric behaviour of oxygen and through the associated chemical reactions which can take place between the oxygen or its reaction products and the contents of the solution. Therefore, oxygen must be removed from the analyte prior to polarographic analysis (199).

Oxygen is reduced at the dropping mercury electrode in two stages. The first stage involves the reduction of oxygen to hydrogen peroxide and /or hydroxide ion.

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \quad \text{(acid media)}$$

$$O_2 + 2H_2O + 2e^- \rightarrow H_2O_2 + 2OH^- \quad \text{(neutral or alkaline media)}$$

$$E_{\frac{1}{2}} \cong -0.05 \text{ V vs. SCE}$$

The second stage involves the reduction of oxygen to hydroxide ion or water.

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \quad \text{(acid media)}$$

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \quad \text{(neutral or alkaline media)}$$

$$E_{\frac{1}{2}} \cong -0.5 \text{ V to } -1.3 \text{ V vs. SCE}$$

The hydrogen peroxide produced can function both as an oxidising agent and a reducing agent. More significantly, pH changes can occur in the vicinity of the DME due to the electro-reduction of oxygen. The resultant increase in pH in the vicinity of the DME can precipitate heavy metal ions and thus diminish their diffusion currents. Also, those species (i.e. organics) whose reduction involves hydrogen ions will be

adversely affected due to the localised increase in pH at the DME.

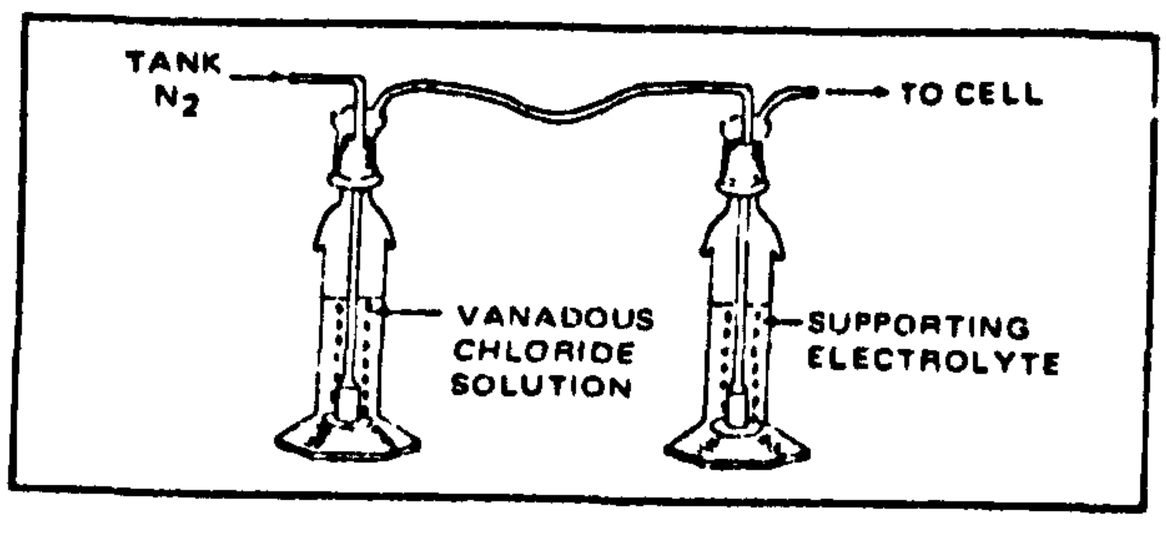
Deaeration is also necessary to prevent the following reaction :-

$$O_2 + 2Hg + 2Cl^- + 2H_2O \rightarrow H_2O_2 + Hg_2Cl_2 + 2OH^-$$

The above non-electrochemical process, which can occur in neutral unbuffered metal salt solutions containing metallic mercury and dissolved oxygen, require that the latter be removed by rigorous deaeration procedures. The most convenient method of removing oxygen is to deaerate the analyte solution with pre-purified nitrogen gas; using an oxygen scrubbing system to remove the last traces of oxygen.

Vanadous chloride solutions can be used for oxygen scrubbing, as shown in Figure 10.

Figure 10



These solutions are prepared from ammonium metavanadate and concentrated hydrochloric acid. Amalgamated zinc is added to the solution to reduce the vanadium to the +2 state. The nitrogen gas brings the solution into intimate contact with the amalgamated zinc and causes the reduction to take place (199).

Polarographic techniques are highly applicable to trace analysis

and are particularly useful for the determination of inorganic constituents of water. Not only is the sensitivity of polarography competitive with other techniques, but its selectivity permits the determination of many constituents without prior chemical separations. Moreover, the range of concentrations which can be determined makes possible the analysis of all types of water ranging from highly contaminated industrial effluents and mineral springs to water prepared for laboratory use by distillation or ion exchange (198).

Determinations can be performed of inorganic or organic species that are either molecular or ionic, provided they undergo oxidation or reduction at a mercury electrode in the region of potential bounded at the positive limit by the potential of oxidation of mercury in the medium employed, and at the negative limit by the potential at which the supporting electrolyte or solvent is reduced. A precision of $\pm$ 3% to 5% is readily attainable at concentrations between 10^{-2} and 10^{-4} M, and with care a precision approaching $\pm$ 1% can be obtained (194).

The everyday application of electrochemistry in analytical laboratories has increased tremendously in the last 15 years. The increase is largely due to the commercial availability of instrumentation that has made it possible to perform voltammetry, particularly the powerful pulse voltammetric techniques, conveniently and inexpensively. With the availability of instruments capable of performing normal pulse polarography (NPP) and differential pulse polarography, the pulsed techniques have taken over for most practical polarographic laboratory analyses (166).

Pulse polarography

The development of pulse polarography by Barker et al. extended the sensitivity of determination of many ions to the 10^{-7} M region, and in some favourable cases, to the 10^{-8} M level (198).

In pulse polarography, a potential pulse is applied to the mercury electrode near the end of its life, with the drop being held at the initial potential during the growth period. Each succeeding drop has an increasing pulse applied to it, with the rate of increase being determined by the selected scan rate. The pulse is applied to the drop for 57 ms before the drop is dislodged. The current is sampled for the final 16.7 ms of the pulse. A voltage analogue of the current level is stored in the memory until the next pulse is applied. The shape of the resulting polarogram is similar to that obtained by means of current-sampled (Tast) dc polarography (196). Since the charging current is allowed to decay before the measurement of the faradaic current, the sensitivity is greater than that of conventional polarography (198).

Differential pulse polarography (DPP)

In DPP, pulses of equal amplitude are superimposed upon a linear potential ramp. The current is sampled immediately before the pulse is applied and just before the end of the pulse. These currents are "differenced", and when the difference current is plotted vs. potential a peak-shaped current response curve is generated.

As seen in Table 19a, the sensitivity of differential pulse lies between that of dc polarography and normal pulse polarography, but the detection limit of DPP is better than those obtained with the more sensitive NPP. The reason is that DPP discriminates more effectively against charging currents (166).

Table 19a : Typical sensitivity and detection limit for DCP, NPP & DPP (166)

Technique	Sensitivity (μA/mM)	Detection limit (M)
DCP	5	10^{-5}
NPP	30	10^{-6}
DPP	20	10^{-7}

b) Anodic stripping voltammetry

Voltammetry is an electrochemical technique in which the current-potential behaviour at an electrode surface is measured. While polarography is voltammetry at a DME (196), stripping voltammetry is a two-step technique in which the first step consists of the electrolytic deposition of a chemical species into or onto an inert electrode surface at a constant potential. The electrodeposition step need not be carried to completion, although this approach secures very precise and accurate results under favourable conditions, it suffers from the drawback of being rather lengthy. Alternatively, pre-electrolysis can be carried out under reproducible conditions for a certain time interval, so that the amount of substance deposited at the electrode is a reproducible fraction of the total initial amount of substance in the solution (200). The preconcentration step can involve either an anodic or cathodic process. The most common use of stripping voltammetry involves a cathodic process in which a metal ionic species is reduced from the solution onto a mercury electrode, resulting in the formation of an amalgam. The second step consists of the application of a voltage scan to the electrode which causes electrolytic dissolution, or stripping, of the various species in the amalgam or film back into solution (201). The resulting stripping voltammogram shows peaks, the heights (or areas) of which are (generally) proportional to the concentrations of the

corresponding electroactive metal ions. The potentials of these have the same qualitative interpretation as their half-wave potentials in polarography (194).

The sensitivity of stripping voltammetry is attributable to the preconcentration that takes place during deposition (201) and is limited, theoretically only by the length of time over which the instrument and electrode system can be kept stable so that deposition may continue, as well as the degree of reproducibility and control which can be exercised over the deposition process. It is theoretically possible to determine solutions well below 10^{-11} M concentrations by this technique through the use of long deposition times. However, instrument instabilities and electrode malfunctions may be a problem when experiments last many minutes, and, more importantly, the excessive times involved can cause all of the analyte to disappear as it is adsorbed on the walls of the vessel (195). As little as 10^{-8} M cadmium has been determined with a precision of $\pm$ 3% using a 15 minute pre-electrolysis time and linear scan rate of 21 mV/s. Because the standard addition method is usually used for evaluating the unknown concentration, the reproducibility will be the same as the precision. By extending the pre-electrolysis time to 60 minutes, the sensitivity is extended to 10^{-9} M but deviations become about 10-20% at this concentration level (194). Sensitivities of the order of 10^{-9} M can, therefore, be obtained and the technique is often used for metal analysis at these levels (195).

Unlike polarography, the DME is not used in stripping voltammetry. The electrode must be stationary. The ideal working situation should have a reproducible surface and area with low residual current (201). The original electrode used for the technique and that most commonly associated with it is the hanging mercury drop electrode (HMDE) (195). The entire stripping voltammetry experiment i.e. a deposition step (preconcentration

step), an equilibration step (material in or on the electrode assumes a new and equilibrium distribution) and an analysis step (stripping step) is performed on one mercury drop (202,201). That drop is then dislodged and a new drop is dispensed for the next experiment (201). The surface of the drop is perfectly smooth and is constantly renewed by the dislodgement process. With solid electrodes, frequent polishing or cleaning is necessary in order to regenerate the original surface. Because the size of the mercury drop is very small, only an extremely low portion of the sample actually reacts during an analysis, i.e. the amount reacting is insignificant, and voltammetry is therefore considered a non-destructive technique.

The high hydrogen overvoltage of mercury allows the analyst to reach more negative potentials than any other metal electrode before the reduction of hydrogen ions begins - the current of which would obscure the current produced by the analyte species.

Mercury behaves as an inert electrode in most solutions, and reacts specifically with a limited number of anions and organics, allowing the analysis of these materials in the presence of other species (203).

Disadvantages of the HMDE are :-

i) a low surface area-to-volume ratio (177)

ii) only minimal stirring of the electrolyte is possible during deposition, otherwise the drop is distorted or dislodged (177)

iii) it is difficult to maintain a stable drop for times of 30 minutes (195)

iv) diffusion of the species of interest into the electrode takes place to a significant degree during the course of the deposition, so that not all the material which has been reduced is available for oxidation when the potential scan is applied (195)

v) there is a limited anodic range. The oxidation of mercury occurs at about +0.4 V, preventing the analysis of materials that are oxidised at more positive potentials (203)

vi) Mercury is a toxic material (203).

Anodic stripping voltammetry at the HMDE can be complicated by intermetallic formation inside the mercury drop (204). The effect of formation of intermetallic complexes in stripping analysis is to reduce the height of the dissolution wave of one of the electroactive species. In a number of cases, a new peak for dissolution of the intermetallic compound is observed (205). A frequently noted example is the interaction of copper and zinc to form a copper-zinc intermetallic species (206); this was first recognised by Kemula et al. (207). One approach to alleviate the problem is to add excess gallium to form preferentially the copper-gallium intermetallic species which has a higher formation constant than that of copper-zinc compounds (206,208). The problem caused by the formation of copper-zinc intermetallic compounds can be avoided by depositing at potentials which allow determination of these two metals separately (206). At the HMDE the interference effects due to the formation of the Cu-Zn compound usually only occur when excessive preconcentration from samples of very high concentration (i.e. > 10 mg/l (201)) is attempted (206,204).

The presence of surface-active substances can have a marked effect on the voltammetric response, owing to the adsorption of such compounds at the electrode surface (209). Adsorption of humic materials on mercury electrodes has been reported. In some extreme cases adsorption of complexing agent or organic matter can cause several effects such as erratic drop behaviour, inhibition of the electrode process, catalytic current, shift of peak potentials, split or drawn-out waves (210).

A major problem in stripping analysis is the presence of trace impurities in the supporting electrolytes which interfere with the determination of very small amounts of material (211). Purification of reagents and careful cleaning of glassware are clearly necessary (204).

The inherently high degrees of accuracy and precision of ASV stem from its application of Faraday's law. Comparative studies with other analytical techniques, such as various atomic absorption procedures, have emphasised the particular reliability of ASV in trace metal analysis for all types of environmental matrices, especially in various types of natural waters (212) and aerosols (213). The technique has been applied to both pharmaceutical and environmental chemistry (214). Moreover, the special property of ASV to be species sensitive renders the technique an efficient tool in speciation studies of toxic trace metals dissolved in natural waters (212).

Differential pulsed anodic stripping voltammetry (DPASV)

Differential pulsed anodic stripping voltammetry (DPASV) differs from dc anodic stripping voltammetry only in that the oxidation process is studied through the use of the pulse-modulated ramps (195). After the deposition and equilibration steps, a waveform consisting of a positive-going linear ramp upon which a small amplitude pulse has been superimposed, is applied to the working electrode (196). The resulting voltammogram has the shape of a sharp maximum and the position of the top of this maximum on the potential axis gives approximately the half-wave potential (i.e. characterises the quality of the depolariser) (177).

In DPASV all the considerations of the dc stripping voltammetry case apply. However, the far greater sensitivity and signal processing capabilities of the pulsed-modulated detection technique permit significantly higher instrument sensitivities to be used and thus allow either much shorter deposition times or much lower instrument gains. Under these circumstances, deposition times are kept to a few minutes so that electrode instabilities are less of a problem, and diffusion into the body of the electrode is less important. Similarly, the

differential pulse scan rate of 5 mV/s or so is such that the system remains in equilibrium with the electrode, except for the effects of the pulse potential change, so that greater reproducibilities may be obtained (195). The sensitivity of the differential pulse technique arises as a result of the potential wave form and data acquisition timing discriminating against charging current contributions (215).

DPASV suffers from two major drawbacks that limit its effectiveness in natural water matrices. First, DPASV disturbs the equilibrium between free and complexed metal ions; dissociation of metal complexes often occurs during the plating step. Therefore, the measured stripping current is composed of two parts : the diffusion current caused by dissolved, hydrated metal ions, those that were not part of complexes; and the kinetic current which arises from metal ions that have just dissociated from complexes. A measure of only the concentration of hydrated metal ions requires that the kinetic current be subtracted from the stripping current. This differentiation is theoretically and experimentally difficult (216). Secondly, organic matter may interfere causing difficulty in the interpretation of results (216,210).

DPASV has been applied to solving numerous trace metal analysis problems in a variety of matrices such as water and waste water (217), soils (218), rock (219), food (220), particulate matter and plankton (221) and raw agricultural crops (222).

DPASV at a rotating disk electrode

In order to avoid some of the problems encountered with the HMDE, solid electrodes of gold, silver, platinum, bismuth and carbon (in its various forms) have been used. The noble metals exhibit a tendency to form intermetallic compounds; to avoid this, the carbon electrode is often now used (223). Graphite also has the advantage that its potential

range is about +1.3 to -1.3 V at pH values between 1 and 7, and from about +1.0 to -1.6 V at pH 10 (197). However, many types of graphite are sufficiently porous for the analyte solution to creep into the substrate, which results in a constantly changing electrode area and poor precision. Wax impregnation prevents solution creep but reproduction of a constant electrode surface area becomes even more difficult (223). Pyrolytic graphites and glassy carbon do not require impregnation (200), but all solid electrodes suffer the same disadvantages that during the deposition step the surface of the electrode and the activity of the deposited metal film are continuously changing. These changes result in irreproducible, and often multiple or split stripping peaks (223).

In electrolysis with solid electrodes, a film is formed on the electrode surface and the situation is more complicated than in the case of amalgams. The process of formation and dissolution of a surface film are governed by more complex parameters, e.g. the structure and surface energy of the electrode, surface catalytic effects, the structure of the deposit formed in the analysis of mixtures.

Interferences are frequently encountered and the attainable sensitivity in practical terms is often lower than that reached with mercury electrodes. To obtain reproducible results, a constant active surface area of the electrode and its reproducible renewal must be ensured (200). Recent efforts have aimed at developing thin film mercury electrodes (TFMEs) (195).

The theory of linear ramp stripping from the TFME was developed by De Vries and Van Dalen (224). The peak current is a function of the mass of analyte present, rather than of its concentration in the mercury film. The scanning rate is the primary stripping parameter controlling peak height, shape and resolution. Unfortunately, rapid scanning rates generate large non-faradaic capacitative currents, which constitute a

background which is difficult to measure, and hence makes a major contribution to poor precision. These effects are minimised by the use of DPASV (223).

The TFME deposited on a solid substrate avoids the limitations of the HMDE and the extensive preparation needed to obtain reproducible results with the carbon electrode. The technique of depositing a TMF 'in situ' on a rotating solid substrate either before or during pre-electrolysis of the analyte metal has emerged as the most reproducible approach (223). Although other electrode materials may be used, glassy carbon usually gives good results (201).

Higher sensitivity is achieved with a TFME, owing to a higher value of the surface area to volume ratio. Also peak resolution is better since metal diffusion inside the electrode is supressed and narrower peaks result. Such a thin-layer electrode can be rotated, e.g. in the form of a rotating disk electrode (RDE) (200).

Once the TFME is generated, it must be protected from oxygen to prevent oxidation of the film. Also, because the layer of deposited mercury is extremely thin, the use of the TFME should be limited to analyte concentrations less than 10^{-7} M (201). Care must also be taken in that the time scale of the dc stripping technique involve massive depletion of the solution, when extremely dilute solutions are being investigated, and exhaustive stripping of the mercury film during the detection process. Thus, the reproducibilities of the whole process will be subject to modification by factors which can affect these massive, exhaustive processes, such as the degree of constancy of the stirring rate, the exact thickness of the film, and the amount of exposure, if any, of the substrate (195).

Because the same electrode surface is used for repetitive analyses, the condition of the surface is a major consideration. Steps must be

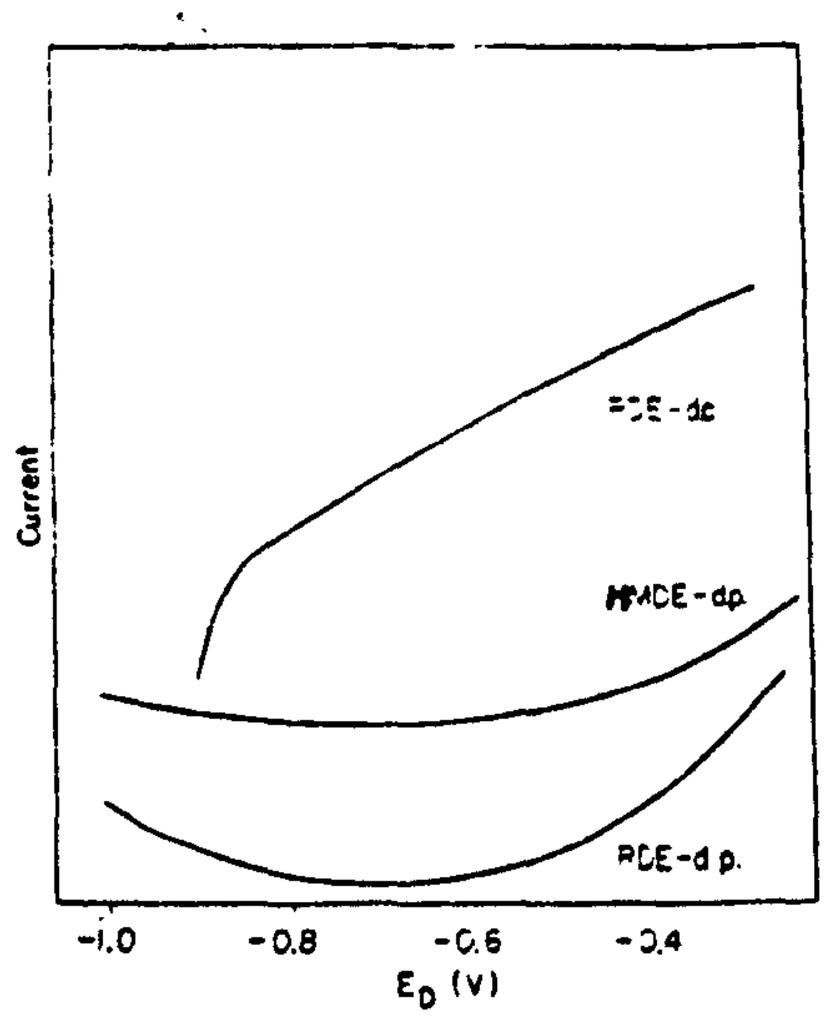
taken to ensure that the surface of the TFME is as reproducible as possible. Procedures that have been used successfully include physically cleaning the surface, applying a conditioning potential, or cycling the potential between empirically determined values. Failure to guarantee a consistent surface may give rise to irreproducible results, since the current due to a particular analyte concentration is dependent upon a reproducible electrode surface (201).

DPASV at a TFME can be complicated by intermetallic formation inside the mercury film. When metals such as zinc and copper are present in high concentrations there is a tendency for a zinc-copper intermetallic compound to be formed when these metals are deposited into the mercury film (201). Although the zinc stripping wave is depressed less when a thicker film is used, the interference is still pronounced. Shorter deposition times decrease the magnitude of depression for the zinc wave, but the interference is still observed, and depends on the Cu^{2+}/Zn^{2+} ratio (206).

The TFME is also affected by the presence of organic matter in the analyte solution. Furthermore, the Hg^{2+} ions added to the solution (in order to form the mercury film in situ) can displace other elements from their complexes and thus increase the electroactive ion concentration. Consequently, the possible presence of Hg^{2+} ions must be mentioned when results obtained by different methods are compared (225).

The shape of the base-line can influence the measurement of peak height, especially when sub-ug/l concentrations are being determined. In DPASV, the base-line appears as a curve, which is more pronounced with a TFME than with an HMDE. Thus, DPASV at an HMDE allows the more accurate peak-height measurement. The base-line shapes for an RDE in the dc ASV and DPASV modes and for an HMDE in the DPASV mode are shown in Figure 11 (225).

Figure 11 : Base-line shapes for the following methods : HMDE-DPASV, RDE-dc ASV and RDE-DPASV



The detection limits and sensitivity of these three methods are given in Tables 19b and 20 (225).

Table 19b : Detection limits for RDE-dc ASV and DPASV and HMDE-DPASV (µg/l)

Element	RDE dc ASV	RDE DPASV	HMDE DPASV
Cd	0.02	0.002	0.02
Pb	0.02	0.002	0.05
Cu	0.02	0.004	0.05

Table 20 : Sensitivity for RDE-dc ASV and DPASV and HMDE-DPASV ($\mu A/cm^2/\mu g$) (225)

Element	RDE dc ASV	RDE DPASV	HMDE DPASV
Cd	4	12	0.6
Pb	5	10	0.6
Cu	4	6	0.4

The TFME is recommended only when maximum sensitivity is required. Because of the care required to produce consistent results, the TFME cannot be considered appropriate for routine analytical purposes (201). The easiest method to use is DPASV with an HMDE. The HMDE used in the differential pulse mode is the best adapted for the measurement of traces of metals at µg/l concentrations. Yet, for lower concentrations, RDE-DPASV is better (225); metals have been determined in seawater using a TFME at concentrations in the order of 1 ng/l (201).

III. Gel Chromatography

Gel chromatography is a form of liquid chromatography in which solutes are separated principally according to their molecular size. Although the technique was initially applied to the separation of very small molecules from very large ones, e.g. salts from proteins (161), it was soon found to be a general method for the analytical and preparative separation of mixtures of macromolecules, and for their characterisation. The same basic concept is known by a variety of names : gel filtration, molecular sieve chromatography and exclusion chromatography.

An important advantage of gel chromatography is that within reasonable limits the separation will be largely independent of the eluent's exact composition e.g. pH and ionic strength. This prevents the destruction of the biological activities of the substances to which the method is best suited. The sample is carefully applied to the end of the sorbent column as a narrow, sharp zone. An eluent is then passed through the column. The effluent is monitored e.g. by UV absorption, it is not necessary to change the eluent composition in order to elute all the components of the sample. During their passage through the column, large molecules are confined to the mobile phase, because they are unable

to diffuse into the porous gel particles. They are, therefore, eluted in the void volume. Blue Dextran 2000, a blue coloured polysaccharide of high molecular weight, can be used to determine the void volume. Smaller solutes enter the gel particles to a greater or lesser extent and are thereby separated from each other and from the molecules travelling in the mobile phase. Elution is complete when a volume of eluent equal to the sorbent volume has passed through the column. The solutes are eluted in order of decreasing molecular size. After complete elution of all solutes, the sorbent may be used directly for a new separation with the same eluent. If it is necessary to change the eluent, this can be done by passing two or three-column volumes of new eluent through the column.

The ideal sorbent for gel chromatography has a carefully controlled and perfectly reproducible porous structure. The range of pore sizes in a given sorbent governs its separation range, and a compromise must be struck between a wide range of pore sizes giving rather poor resolution, and a narrow range, giving excellent resolution, but having a limited sphere of application. Therefore, a series of sorbents is required, each of which separates solutes over a characteristic size range.

Table 21 gives some of the physical properties of the cross-linked dextran gels commercially available under the name of Sephadex.

Sephadex, being covalently cross-linked dextran, is insoluble in all solvents unless chemically degraded; however, the gels of high water regain contain a certain amount of dextran which is not cross-linked and which is therefore extracted during swelling and equilibration.

Table 21 : Some physical properties of the Sephadex G-types (226)

Sephadex type and grade	Dry bead diameter μ	Fractionation range (molecular weight) Peptides and globular proteins	Dextrans	Bed volume ml/g dry Sephadex
G-10	40-120	- 700	- 700	2-3
G-15	40-120	- 1,500	- 1,500	2.5-3.5
Coarse	100-300	1,000- 5,000	100- 10,000	
Medium	50-150			
Fine	20- 80			
Superfine	10- 40			
G-50 Coarse	100-300	1,500- 30,000	500- 10,000	9-11
Medium	50-150			
Fine	20- 80			
Superfine	10- 40			
G-75	40-120	3,000- 80,000	1,000- 50,000	12-15
Superfine	10- 40	3,000- 70,000		
G-100	40-120	4,000-150,000	1,000-100,000	15-20
Superfine	10- 40	4,000-100,000		
G-150	40-120	5,000-300,000	1,000-150,000	20-30
Superfine	10- 40	5,000-150,000		18-22
G-200	40-120	5,000-600,000	1,000-200,000	30-40
Superfine	10- 40	5,000-250,000		20-25

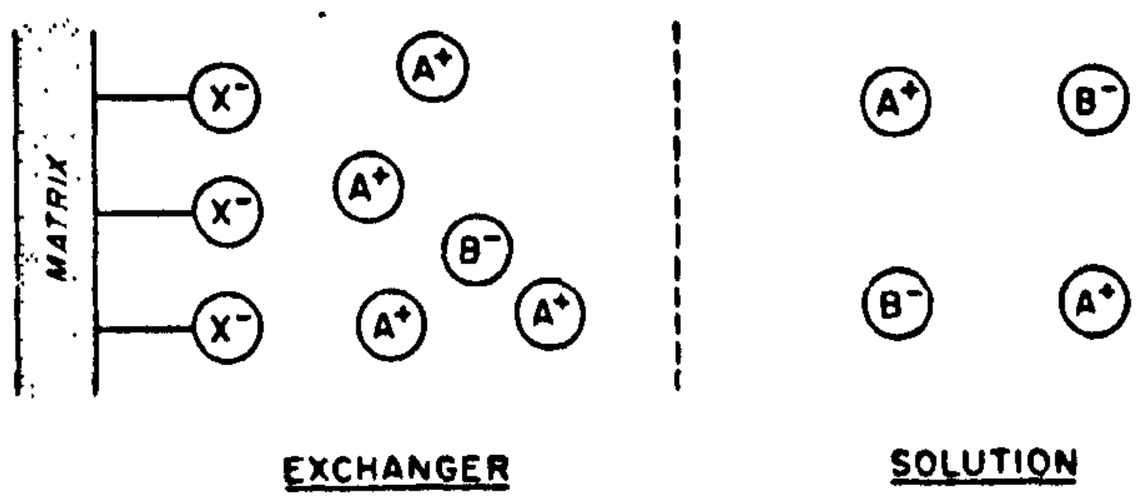
Their stability in organic solvents and in aqueous solutions between pH 2 and pH 12 is excellent.

Gel chromatography, in combination with ion-exchange chromatography, forms a powerful and versatile tool for analytical and preparative chemistry (161).

IV. Ion Exchange Chromatography

Ion exchange chromatography has been used for several decades to separate and isolate proteins and other biomacromolecules (227). An ion-exchange solid consists of a polymer or a material of high molecular weight that is insoluble, yet permeable to the solution with which it is in contact, and with whose ions it will exchange. The polymer skeleton, or matrix, carries functional groups that are the 'fixed ions'. Balancing the charges of the fixed ions are the 'counter-ions', the mobile ions that can change places with ions of similar charge in solution. When exchanger is in contact with an electrolyte solution, it absorbs molecules of the solvent and also ions whose charge has the same sign as the fixed ions. These ions are called co-ions. The situation is shown in Figure 12.

Figure 12 : Schematic representation of an ion exchanger in equilibrium with a solution. Ions A^+ are counter-ions; B^- are co-ions.



When cellulose fibres are mixed with aqueous sodium hydroxide and then warmed with chloroacetic acid, some of the hydroxyl groups of the cellulose are converted to the group $-OCH_2COONa$. It is possible to prepare an insoluble cellulose product whose ion-exchange capacity is about 1 meq/g. By modification of the chemical treatment, other

functional groups can be introduced.

Ion exchangers based on cellulose have their fixed ions on the surface of fibres or easily accessible in large pores. Such exchangers are well suited to the absorption of large molecules, including proteins and nucleic acids. They are also used for separation of inorganic ions, though their ion exchange capacities are much lower on a volume basis than those based on polymeric exchangers (228).

V. Speciation Schemes

Speciation of an element is the determination of the individual physico-chemical forms of that element which together make up its total concentration in a sample (229).

The importance of chemical speciation of metals has been recognised for over a decade. It is now known that the bioavailability of a metal is not regulated by its total concentration but usually by the concentration of the uncomplexed species (230). Sorption of metals onto particulate matter, and therefore metal transport, also depends on metal speciation (230). The availability of heavy metals in the environment markedly influences their effect on higher plants and micro-organisms. Measurements of the total metal concentrations of a soil or sediment are therefore unlikely to reflect the amount actually available to the biota (231). It is well established that speciation measurements are necessary for the study of toxic metals towards aquatic organisms and for an understanding of trace element transport in rivers and estuaries (229).

Recognition of these facts has led to the development of methods for determining the concentration of complexed and uncomplexed species. Some rely on the use of chemical extraction schemes, while others use

phvsical methods of separation (230,229,232).

a) Chemical extraction

The partitioning of trace elements by selective dissolution allows information to be obtained about the associations and origin of elements and permits the biological and physico-chemical availability of the elements to be assessed (233). Since the strength of metal retention may be as important as the quantity retained in assessing agronomic or environmental mobility (234) a wide range of extractants have been evaluated to determine the concentration of available metal, although no consensus has been arrived as to which is the best extractant (231). A number of chemical extractants and sequential extraction schemes have been proposed (235). For example, Lum and Edgar applied the scheme outlined in Figure 13 to sediments (236).

Duddridge and Wainwright carried out extractable metal analysis on sediment using three separate extractants :-

i) 0.1 M ammonium acetate (50 ml) pH 7 and pH 4.8

ii) 0.1 M EDTA (50 ml)

iii) Deionised water (50 ml) pH 6 and shaken for 3 hours. They concluded that EDTA was the best extractant for all metals; while deionised water was very poor (231).

Slavek et al. studied the ability of a range of electrolyte solutions (shown in Table 22) to release metal ions (Cu, Pb, Cd, Zn) preabsorbed on two samples of humic acid (237).

Figure 13 : Extraction scheme used by Lum and Edgar (236)

	Extractant	Conditions	Fraction
sample	0.75 M LiCl	10 minutes	Readily exchangeable
↓	0.25 M CsCl	room temp.	ions
	60% CH_3OH		
residue	1 M CH_3COONa	pH 5, 5 hours	Carbonate bound -
↓		room temp.	surface oxide bound ions
residue	1 M $NH_2OH.HCl$	2 hours room	Ions bound to Fe-Mn
↓	25% CH_3COOH	temp.	oxides
residue	H_2O_2		
↓	1.2 M CH_3COONH_4-	5 hours 90°C	Organically and sulphide
	- 20% HNO_3		bound ions
residue	HF - HCl - H_2O_2		Ions bound to residual
			phase

Table 22 : Extractant solutions used by Slavek, Wold and Pickering (237)

1 M HNO_3	1 M $MgCl_2$
0.5 M HCl	0.05 M $CaCl_2$
0.5 M HAc	0.05 M NaCl
0.5 M HOx	0.1 M $Na_4P_2O_7$
0.2 M HOx/NH_4Ox	0.005 M EDTA
0.5 M NH_4 Ac/HAc	0.005 M DTPA
1 M NH_4Ac	
1 M NH_4NO_3	

Their results indicated that treatment with mineral acid or a chelating agent released a high proportion of the retained metal ion, however recoveries were never totally quantitative. Concentrated salt solutions displaced about 80% of the retained Cd or Zn, and about half of any Cu or Pb held by the organic matter (237). Similar work was carried out by Watanabe et al. The extractants studied are given in Table 23.

Table 23 : Extractants applied to sediment by Watanabe et al. (238)

conc. HCl and conc. HNO_3
3 M HCl
1 M HCl
0.5 M HCl
6 M HCOOH
4.4 M HAc
0.05 M EDTA
1 M NH_2OH.HCl & 25% HAc

The application of 0.05 M EDTA or 1 M NH_2OH.HCl plus 25% v/v HOAc was found to be effective for evaluating the extent of heavy metal pollution of aquatic sediments for Cu, Pb, Cd and Zn (238).

Miller et al. carried out a sequential extraction of soil using the scheme outlined in Figure 14 (234).

Figure 14 : Sequential extraction scheme of Miller, McFee and Kelly (234)

Extractant		Fraction
H_2O (30 minutes)	⟶	soluble
1 M KNO_3	⟶	exchangeable
0.5 M NH_4F	⟶	adsorbed metal
0.1 M $Na_4P_2O_7$	⟶	organically bound
0.1 M EDTA (pH 7)	⟶	carbonate metal
0.1 $NH_2OH.HCl$ (pH 3, 30 minutes)	⟶	Mn- oxide-bound metal
Citrate-bicarbonate-dithionite (80°C, 15 min)	⟶	Fe oxide-bound metal
1 M HNO_3	⟶	precipitated metal
Conc. HNO_3	⟶	residual metal

A simpler scheme was used by Lau and Wong (239). Samples of roadside dust were subjected to extractants to determine the total, extractable and soluble amounts of heavy metals :

i) 6.5 ml of a mixture of concentrated HNO_3 : concentrated H_2SO_4 : 60% $HClO_4$ = 5 : 0.5 : 1 (boiled for 1 to 2 hours at 180°C) to obtain total metal content.

ii) 1 M ammonium acetate (125 ml, pH 7) shaken at 100 rpm for 1 hour to obtain extractable metal content.

iii) Distilled water (125 ml, pH 7) shaken and stirred at 100 rpm for 1 hour to obtain the water-soluble content.

The scheme also incorporated physical methods of separation in that the dust particles were passed through a series of sieves with apertures of 15, 40, 75, 106, 125, 250 and 500 μm respectively, before chemical

extraction was carried out. They found that dust particles with a size smaller than 125 µm belonged to a homogeneous group and did not have a significant difference in metal contents. In using dust as a parameter to indicate the level of lead contamination, they recommend the determination of the extractable metal content for the particle size of 125 µm (239).

Many other extraction schemes have been used (240). A summary of the sequential approach proposed by some researchers is given in Table 24 (237).

A scheme that has found wide application is the one proposed by Tessier et al. The scheme allows the selective extraction of five fractions (241) :-

1. Fraction 1. Exchangeable. Changes in ionic composition are likely to affect the sorption-desorption process (219).
2. Fraction 2. Bound to carbonates. Significant trace metal concentra tions can be associated with carbonates and are susceptible to changes of pH (242).
3. Fraction 3. Bound to iron and manganese oxides : these oxides are excellent scavengers for trace metals and are thermodynamically unstable under conditions of low Eh (92).
4. Fraction 4. Bound to organic matter. Trace metals may be bound to various forms of organic matter. Under oxidising conditions organic matter can be degraded, leading to a release of soluble trace metals (95).
5. Fraction 5. Residual. When the first four fractions have been removed, the residual solid should contain mainly primary and secondary minerals, which may hold trace metals within their crystal structure (241).

Table 24 : Extraction sequences for the subdivision of the total metal content of soils (237)

Cu	Zn,Mn,Cu	Cd,Co,Cu,Ni,Pb,Zn Fe,Mn	Cu,Zn,Fe,Mn,Mo
	"Available" or "Exchangeable" ($MgCl_2$) (or NaAc)		Sulphides and bound to organic matter (NaOCl)
Weakly bound, specific sites (HAc)		Bound to carbonates (HAc/NaAc)	Adsorbed ions. soluble carbonates (HCl)
Bound to organic matter ($Na_4P_2O_7$) (H_2O_2)			
	Bound to hydrous oxides of iron and manganese (HOx/NH_4Ox)		($NH_2OH.HCl$)
		($Na_2S_2O_4$/citrate/HCO_3^-)	
		Bound to organic matter (H_2O_2)	
Residual (lattice) components (HF)	Sand (totals) Silt (totals) Clay (totals) Soil (totals) (HCl/HNO_3/HF)	Residual solids (HCl/HF)	Silicate minerals (HNO_3/$HClO_4$)

The selective extractions were conducted in polypropylene centrifuge tubes to minimise losses of solid material. The scheme is outlined in Figure 15.

Figure 15 : Tessier et al. extraction scheme (241)

Fraction	Extraction procedure
Exchangeable	Room temp. 1 hour 8 ml 1 M $MgCl_2$ pH 7 or 1 M NaOAc pH 8.2 continuous agitation
(Residue)	
Carbonate	Room temp. 8 ml 1 M NaOH (to pH 5 with HAc). Continuous agitation.
(Residue)	
Fe-Mn oxide	20 ml 0.04 M $NH_2OH.HCl$ in 25% (v/v) HAc. 96 $\pm$ 3°C. Occasional agitation.
(Residue)	
Organic	3 ml 0.02 M HNO_3 & 5 ml 30% H_2O_2 (to pH 2 with HNO_3) 85 $\pm$ 2°C. 2 hours occasional agitation. 3 ml H_2O_2 (30% & pH 2) & repeat heating for 3 hrs. When cool, 5 ml 3.2 M NH_4OAc in 20% (v/v) HNO_3; dilute sample to 20 ml. Continuous agitation 30 min.
(Residue)	
Residual	HF-$HClO_4$ digestion.

Many reagents including ammonium acetate (243), sodium acetate and magnesium chloride (241) have been employed to liberate exchangeable metals. Tessier et al. found that $MgCl_2$ solution was an effective reagent for desorbing specifically adsorbed trace metals.

By using 1 M NaOAc/HAc (pH 5) the carbonate fraction is dissolved without affecting the organic carbon and free iron concentrations. At lower pH values there is partial attack on Fe and Mn oxides (241).

The leaching of iron and manganese oxides can be accomplished by reducing these metals to their ferrous and manganous forms respectively. Agents to do this must also be capable of maintaining the released metals in solution. The solutions 1) hydroxylamine hydrochloride-acetic acid and 2) sodium dithionite-citrate were investigated. The latter was rejected for the following reasons :-

i) precipitation of trace metals occurred, probably due to the formation of sulphide as a result of the disproportionation of dithionite;

ii) dithionite is highly contaminated with zinc and its purification by a chelation-extraction procedure proved difficult;

iii) for FAAS, burner clogging occurred due to the high salt content of the extraction solution (241).

Hydrogen peroxide in an acid medium may not be sufficient to completely oxidise all forms of organic matter. More efficient methods exist (e.g. concentrated nitric acid separately or in combination with hydrochloric or perchloric acids), but usually suffer from a lack of specificity, i.e. the agents may also partially attack silicate lattices. Hot hydrogen peroxide in a nitric acid medium was, therefore, adopted (241).

To ensure complete decomposition of silicates, Tessier et al. selected an $HF-HClO_4$ digestion procedure which had the advantage of not interfering with AAS readings, as would be produced if a fusion salt decomposition was used (241).

For this scheme, the precision was found to be generally low when the concentration approached the detection limit but improved for higher concentrations; coefficients of variation of 10% or lower were observed for metal concentrations 5 times greater than the detection limit (241).

The scheme has been modified for use with street dusts and soils (235) and with estuarine and oceanic sediments (233).

Farago et al. carried out a sequential extraction of copper from the leaves and roots of Armeria maritima collected from the copper-impregnated Dolfrwynog Bog. The scheme allows the plant material to be broken down into the following segments :-

pigments
soluble protein
soluble pectates
low molecular weight materials
polar low molecular weight materials
proteins and amino acids
insoluble pectates
protopectates
∝-cellulose and lignin
hemicellulose
polysaccharides
lignin
∝-cellulose

The scheme thus permits the identification of metal binding sites within the plant (126).

b) Physical separation

Most trace metal speciation schemes developed to date use ASV, ion exchange with Chelex-100 resin (18,244), filtration (245), ultrafiltration or dialysis (18,244), centrifugation (246) or various combinations of

these techniques (244).

In the analysis of natural waters, filtration through a 0.45 micron pore size membrane filter is commonly used to differentiate between particulate and soluble metals (247). The distinction between particulate and dissolved is arbitrary and colloidal material can pass through 0.45 µm filters (245). But the different size fractions may contain trace metals in particular associations. Hence, the size distribution of trace metal concentrations can reflect the relative importance of these different metal species (232).

Laxen and Harrison have applied the size distribution approach to the speciation of trace metals in freshwaters, using filtration through various pore size Nucleopore filters to differentiate the size fractions (248) (Table 25).

Laxen and Chandler compared filtration of freshwaters through screen filters (Nucleopore filters in which particles are trapped on the filter surface) and depth filters (Sartorius, in which particles may be trapped within the depth of the filter as well as on the surface). Screen filters provided the more accurate size fractionation, as long as the filter was not allowed to clog. No benefit from stirring the sample during filtration was observed (245).

Millipore filters have been found to exhibit the best selectivity characteristics. However, for Nucleopore polycarbonate filters, the effective pore size remains relatively constant with filter loading until the pores are blocked. Nucleopore filters are more easily over-loaded than Millipore filters (232).

Filtration through 0.45 µm remains the most often used method. Care must be taken with commercial equipment to prevent significant retention of metals from unacidified water samples (249). Florence reported that all-glass Millipore apparatus is suitable for natural waters since no

Table 25 : Metal forms in water (248,232)

	1 nm			10 nm		100 nm	1000 nm
Size	SOLUBLE			COLLOIDAL			PARTICULATE
Metal Species	Free metal ions	inorganic ion pairs: organic chelates	organic complexes	bound to high molecular weight organic material	Adsorbed on colloids	with organic particles & remains of organisms	Adsorbed on precipitates, co-precipitates
Example	Cd^{2+}	$PbHCO_3^+$ Pb-EDTA	Zn-FA	Pb-HA	Pb-Fe(OH) $Pb-MnO_2$	Pb-organic solids	Cu-clay $PbCO_3$

adsorptive losses of cadmium, copper, lead and zinc were observed (18). Adsorptive losses in size fractionation schemes can be minimised if filtration is carried out in parallel rather than in series. The procedure may not be feasible for waters with high particulate loading, but the number of filters through which a water sample must pass should be kept to a minimum (232).

The mode of filtration (pressure or vacuum) may have a large influence since natural water with low buffering capacities can lose significant amounts of CO_2. A pH variation of up to 2 pH units may occur through the membrane accompanied by displacement of solubility equilibria. However, phase separation by any means breaks the pre-existing equilibrium state (if there were any) between water and particulates and induces some chemical evolution (249).

Similar problems to those encountered in filtration techniques can be expected in ultrafiltration, namely size selectivity, contamination and adsorption, but are not so well resolved, particularly with regard to trace metals. Ultrafiltration (filtration with membranes having a nominal pore size smaller than 15 nm) can be used to discriminate further the size continuum of material in natural waters. The retentive capability of an ultrafilter is usually designated by a molecular weight cut-off value. This value refers to the molecular weight of a globular solute which is 90% retained. Such an assignment is only a nominal value because fractionation is achieved by molecular size rather than weight. Ultrafiltration techniques have been incorporated into trace metal speciation studies. Although the absolute molecular weight of the material cannot be determined, suspended matter is crudely fractionated and some trends are evident in the size distribution of trace metals in natural waters (232).

Centrifugation can be used to remove particles from suspension in

natural waters (232,246). Settling rates of particulate matter are a function of not only size but also density. As particulates are not fractionated strictly by size, this complicates the direct comparison of particulate metal concentrations measured following centrifugation as opposed to conventional filtration (nominal pore size 0.4 - 0.5 μm). Small dense particles that settle during centrifugation may pass through a filter (232).

To date, dialysis procedures have had only limited application in physico-chemical speciation studies. Ideally dialysis membranes should be permeable only to species in true solution so that the concentration of free metal cations is the same in both the diffusate and retentate solutions. But in practice, because nominal pore sizes range from 1 to 5 nm (compared to 1-14 nm for ultrafilters), some relatively high molecular weight material may pass through the dialysis membrane. The major disadvantage of dialysis methods is the long time required for equilibrium to be established. The problem is compounded by the fact that the membranes often possess a negative charge causing cations, anions and neutral molecules to diffuse through the membrane at different rates. Therefore the time required to ensure complete equilibration depends on those molecules with the slowest diffusion rates, usually anionic species (232).

Chelex-100 resin was first used for speciation schemes by Florence and Batley (250,251). The resin has a pore diameter of about 2 nm; colloidal particles are therefore excluded from the resin matrix, providing a simple and contamination-free method for separating ionic and colloidally-associated metal (221). Displacement of chemical equilibria may occur so that chelates that are labile enough are displaced (249).

Electrochemical techniques have some distinct advantages for

speciation measurements :-

1) electrochemical methods can be highly sensitive, a property which is essential when analysing natural waters, where the total concentration of a heavy metal is often in the ug/l or ng/l range;

2) the operating conditions of techniques such as polarography and ASV can be adjusted so that only metal complexes with dissociation rates within a desired range are included in the electroactive metal fraction.

Some of the parameters which can be adjusted to achieve this selectivity are deposition potential, electrode rotation (or solution stirring), rate, pulse frequency and potential scan rate (206).

In addition to achieving speciation on the basis of complex lability, polarography and ASV can also be used to distinguish between different valency states of the same element in solution e.g. Fe (II)/Fe (III), Cr (VI)/(III) (229). The determination of ionic alkyl lead species in marine fauna has been carried out, using RDE-DPASV (252). On the whole, ASV with a mercury electrode is the most useful method due to its extremely high sensitivity, convenience even for unpolluted fresh waters, and is easy to use as reliable commercial equipment is available. Nevertheless, very few metals are susceptible to direct and sensitive ASV measurement in water : Pb, Cd, Cu, Zn, Tl, are the most interesting (249).

SECTION 3 : OBJECTIVES

The objectives of the present work were :

i) to identify sources of contamination in the analysis of Cd, Cu, Pb and Zn by DPASV and AAS, and to determine the most suitable methods of purification and/or prevention of contamination;

ii) to determine the analytical precision, reproducibility and detection limits of the HMDE and RDE;

iii) to investigate the speciation of Cd, Cu, Pb and Zn in natural waters by means of sequential filtration, decreasing pH and analysis by DPASV;

iv) to investigate the speciation of Cd, Cu, Pb and Zn in contaminated river sediments by means of sequential filtration, decreasing pH and DPASV;

v) to test the applicability of the Tessier and Farago chemical extraction schemes by the use of model samples;

vi) to relate the heavy metal content of soils, and woodland litter to identifiable fractions by application of a chemical extraction scheme, and therefore understand the availability of these elements for uptake by plants;

vii) to investigate the effect of humic and fulvic acid on Cd, Cu, Pb and Zn;

viii) to investigate the storage of heavy metals within plants, collected from metal contaminated sites, by means of a chemical extraction scheme;

ix) to investigate the uptake and storage of Cd, Cu, Pb and Zn from nutrient solution by plants known to be metal tolerant, by means of a chemical extraction scheme, and thus to indicate possible tolerance mechanisms;

x) to investigate the presence or absence of a metallothionein-like protein in metal tolerant plants.

PART II

EXPERIMENTAL & RESULTS

INTRODUCTION

Since natural waters are an important sink for, and carriers of, trace metals, considerable efforts have been made to study these metals in aquatic media (1,4,244,252). In natural waters trace metals rarely exceed 1 mg/ml in concentration even under highly mineralised conditions (92). It would be unrealistic to try to determine all the possible species in which each element occurs in the water samples under study (1). The simplest ASV procedures for chemical speciation in waters are the determination of labile (i.e. weakly complexed) metal and total metal via an acid digestion (253). By subtracting the labile fraction from the total metal content, the quantity of strongly complexed or "bound" metal can be determined (251). The "bound" metal may be considered as metal bound in inert complexes or other inert forms such as colloidal particles (253).

ASV labile metal determinations at natural pH measure that metal present as :-

i) free ions

ii) simple inorganic complexes

iii) weak organic complexes.

Such determinations are normally conducted in faintly acid solutions, often using an acetate buffer of pH 5. Further acidification should release those metals held in :-

i) stronger organic complexes

ii) organic and inorganic colloids

iii) absorbed on particulate matter (177).

Filtration has become a universally applied first step in the preparation of water samples for trace metal analysis. By tradition this filtration is usually performed with a 0.45 µm membrane filter (245).

A single filtration step is often the only size differentiation used and forms the basis of several speciation schemes (232).

It may be advisable to study the water sample as a whole, since particles larger than 0.45 μm participate in reactions such as adsorption-desorption and thus exert a strong influence on the level and distribution of dissolved trace metals (254).

Filtration through different pore size filters to obtain the various size fractions and their associated metals has been carried out with some success (245,247). For example :-

Size	Associated fraction
"Small"	free ions/inorganic complexes
"Medium"	organic complexes
"Large"	colloids

By using this size differentiation in conjunction with the chemical behaviour of the size groups (e.g. distribution changes due to change in pH), speciation can be carried out.

Care must be taken in any investigation of trace metal speciation, to minimise any possible effects of contamination and metal loss (232). The metal content of the supporting electrolyte is a major limitation in the sensitivity of DPASV. The contamination is derived from reagents, the atmosphere and glassware. Tissue paper and Millipore filters have been found to contain metals (especially zinc) often in appreciable concentrations - zinc concentrations of 48.8 μg/g have been recorded in Kimwipe tissue paper and 2.4 μg/g in a Millipore filter (255,256).

In μg/l level determinations, contamination of the test solution can lead to significant errors. Purification of reagents and glassware preparation must be carried out with care.

For cadmium, copper, lead and zinc, the oxidation current peaks

have been found to be independent of the solution pH in the range from 4 to 7, but beyond 8, the oxidation current peaks decrease sharply. There is some evidence that acetate buffer is a better supporting electrolyte than potassium chloride for analysis of natural waters above pH 7 (257). Florence and Batley using acetate buffers found that pH 5 was the optimum pH for the formation of many complexes although some weak complexes may dissociate and slow desorption of adsorbed cadmium or zinc, but not lead or copper, might occur (258). Zinc determination by ASV at very acid pHs is not possible due to interference from the reduction of hydrogen ions (259).

Buffered solutions as supporting electrolytes have two main advantages :-

1) sample pH may vary; the use of a buffer allows comparison between samples,
2) the pH of the medium is kept constant at a desirable level when the deaeration step is performed.

Sample pretreatment and measurement steps can induce equilibrium shifts - a considerable problem in speciation studies - and thus should be kept to a minimum (249). Acidification, in order to prevent metal adsorption on vessel walls, is obviously not possible. The speciation of cadmium, copper, lead and zinc has been found not to alter significantly within 1-2 months, when stored at 4°C or within 27 days at 25°C (258). Other workers have reported losses of metals due to adsorption on vessel walls (260-262), the adsorption being greater on glass flasks rather than polyethylene bottles (262).

Several mineral acids, such as hydrochloric, nitric, sulphuric, perchloric and hydrofluoric acids individually or as mixtures have been widely used for the determination of "total" metal in samples (263-267).

A disadvantage of sulphuric acid is the possible formation of

insoluble sulphates (264). The sulphate anions tend to interfere with FAAS determinations, by forming salts which are very difficult to dissociate and atomise in the flame (171).

Hydrochloric acid dissolves complex adsorbed and precipitated metals (268) and attacks the lattice structures of certain clay minerals (269) but has little effect on the more inert silicate metals (268).

Nitric acid has been recommended for partial extraction of trace metals due to the fact that all nitrates are soluble and the acid is a good medium for AAS, causing few interferences (177).

Hydrofluoric acid alone and in conjunction with nitric, hydrochloric or perchloric acid is still the favoured agent for the decomposition of silicates.

SECTION 1 : FRESHWATER : METAL SPECIATION STUDIES

1.1 Experimental Preparations

a) Reagents

b) Glassware Preparation

c) Preparation of Stock Solutions

i) stock metal solutions

ii) vanadous chloride solutions

iii) background electrolytes

d) Purification Methods

i) isothermal distillation

ii) cation-exchange resin

iii) other methods of water purification :-

Millipore "Milli-Q" water system

distillation/deionisation

iv) other methods for the purification of background

electrolytes :- solvent extraction

mercury cathode electrolysis

e) Site Description

f) Sample Collection

g) Sample Treatment, Analysis and Results

h) Apparatus and Instrumental Parameters

SECTION 1 : FRESHWATER : METAL SPECIATION STUDIES

1.1 Experimental Preparation

a) Reagents

AnalaR and AristaR reagents were used whenever possible, these were :-

AnalaR and AristaR nitric and acetic acid

AnalaR cadmium nitrate, copper nitrate, lead nitrate and zinc nitrate, ammonium acetate, sodium acetate, citric acid, 0.91 ammonia solution, chloroform, dithizone, ammonium chloride, and ammonium pyrrolidine dithiocarbamate.

For procedures not involving trace metal analysis, "BDH Chemicals Ltd." (BDH) compounds were used. These were :-

Ammonium metavanadate, hydrochloric acid (laboratory reagent grade) and granular zinc; used for the preparation of vanadous chloride solution.

b) Glassware preparation

All glassware was washed with detergent (Teepol), rinsed with tapwater and twice with DDW, before being placed in 25% v/v nitric acid for at least 24 hours. The glassware, on removal from the acid, was rinsed twice with DDW and drained dry.

c) Preparation of stock solutions

i) Stock metal solutions

Stock solutions containing 1000 µg of the metal per ml in HNO_3 (pH ~ 1) were prepared by dissolving the appropriate weights of the metal nitrates in AnalaR nitric acid (5 ml) and DDW, and diluted to a final volume of 500 ml. Acidification at pH 1.5 or less was necessary to ensure that the metals remained in solution (270,271). The standard solutions of cadmium, copper, lead and zinc were prepared from AnalaR $Cd(NO_3)_2.4H_2O$

(99% pure), $Cu(NO_3)_2.3H_2O$ (98% pure), $Pb(NO_3)_2$ (99.5% pure) and $Zn(NO_3)_2.6H_2O$ (98% pure).

ii) Vanadous chloride solutions

A vanadous chloride solution was used for the removal of oxygen from the nitrogen purge gas for DPASV.

Ammonium vanadate (2 g) was boiled with 25 ml of concentrated hydrochloric acid and the solution was diluted to 250 ml with DDW. The solution was placed in two Dreschel flasks. Amalgamated zinc was prepared from 10 g of granular zinc. The zinc was placed in a beaker and covered with DDW. Concentrated HCl (2 drops) was added. Addition of approximately 20 ml of mercury gave rapid amalgamation.

The amalgamated zinc was added to reduce the vanadium. Nitrogen gas (white spot) was passed through the solution in the flasks until a clear violet colour was obtained.

iii) Preparation of background electrolytes

Ammonium acetate pH 7 : A 0.2 M solution of AnalaR ammonium acetate was prepared by dissolving 1.5 g ammonium acetate in 100 ml of DDW.

Sodium acetate pH 5 : A 0.2 M buffer was prepared by mixing 0.2 M acetic acid and 0.2 M AnalaR sodium acetate in the ratio 3:7. (1.6 g sodium acetate in 100 ml DDW and 11.5 ml of 17.5 M acetic acid in 100 ml DDW).

Ammonium citrate pH 3 : The 0.2 M buffer was prepared by dissolving 4.2 g of AnalaR citric acid in 80 ml of DDW. Sufficient ammonia solution was added to the citric acid solution to give a reading of pH 3 $\pm$ 0.2 on an Electronic Instruments Ltd. Model 7050 pH meter. The volume was made up to 100 ml with DDW.

d) Purification methods

i) Preparation of High-Purity Volatile Acids by Isothermal Distillation

Where AnalaR or AristaR acids were unavailable, acids of high-purity were prepared by isothermal-distillation. The method has been described by Vellon and Reamer (272) for the purification of hydrochloric and acetic acids. Purification of hydrochloric acid and ammonia solution has been described elsewhere (204,273).

A large glass vacuum desiccator was thoroughly cleaned and allowed to dry. The flange was not greased and the vacuum port was left slightly open to prevent pressure build up from lifting the desiccator, so providing a closed system.

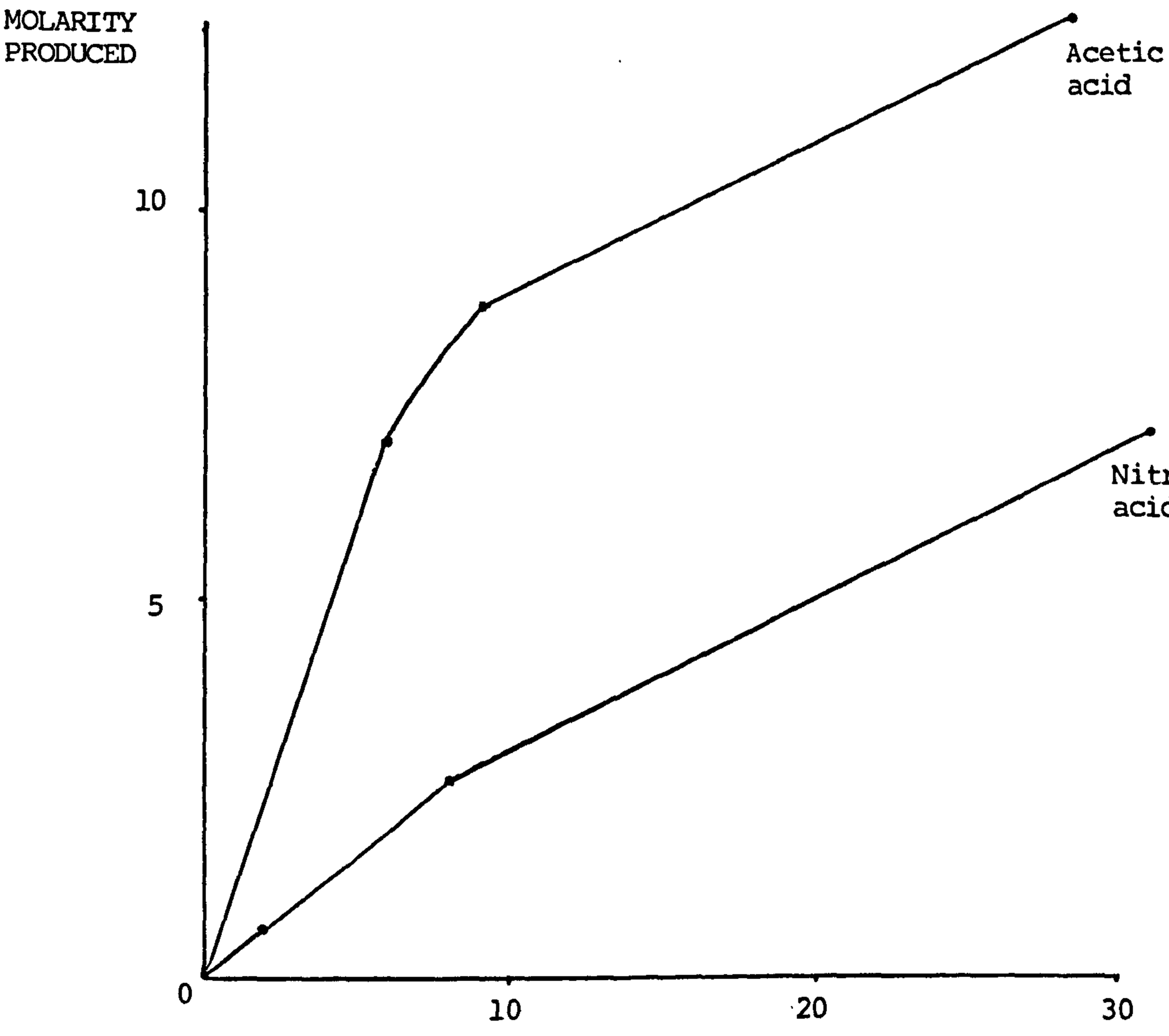
New polypropylene 250 ml beakers were cleaned by soaking in 6 M HNO_3 solution for 1 day and then rinsed several times with DDW. A concentrated reagent grade acid was placed in one beaker and ultrapure water was placed in another beaker. The beakers were placed in the desiccator and allowed to stand within the closed system for the desired length of time. Acid vapours continously condense into the pure water until an equilibrium is obtained. The molarity of the acid in the "water" beaker was checked and a graph of molarity vs. time of exposure was prepared (Table 26, Figure 16). The molarity of the 'distilled acid' can be increased by renewing the reagent grade acid, or by increasing the number of acid containing beakers.

ii) Purification of water and background electrolytes using a cation exchange column

A differential pulse anodic stripping curve for tap water is shown in Figure 17. For the speciation of metals in natural waters by DPASV ultrapure water is required. As can be seen from Figure 17 and

Figure 16 : Preparation of pure acids by isothermal distillation.

Graph of molarity produced against time (days)



(using non-renewed acid, in the ratios of
1 DDW : 3 Acetic and 1 DDW : 6 Nitric)

Figure 17 : Differential pulse stripping curve (HMDE) for Zn, Cd, Pb and Cu in 5% v/v tap water : 0.02 M ammonium citrate

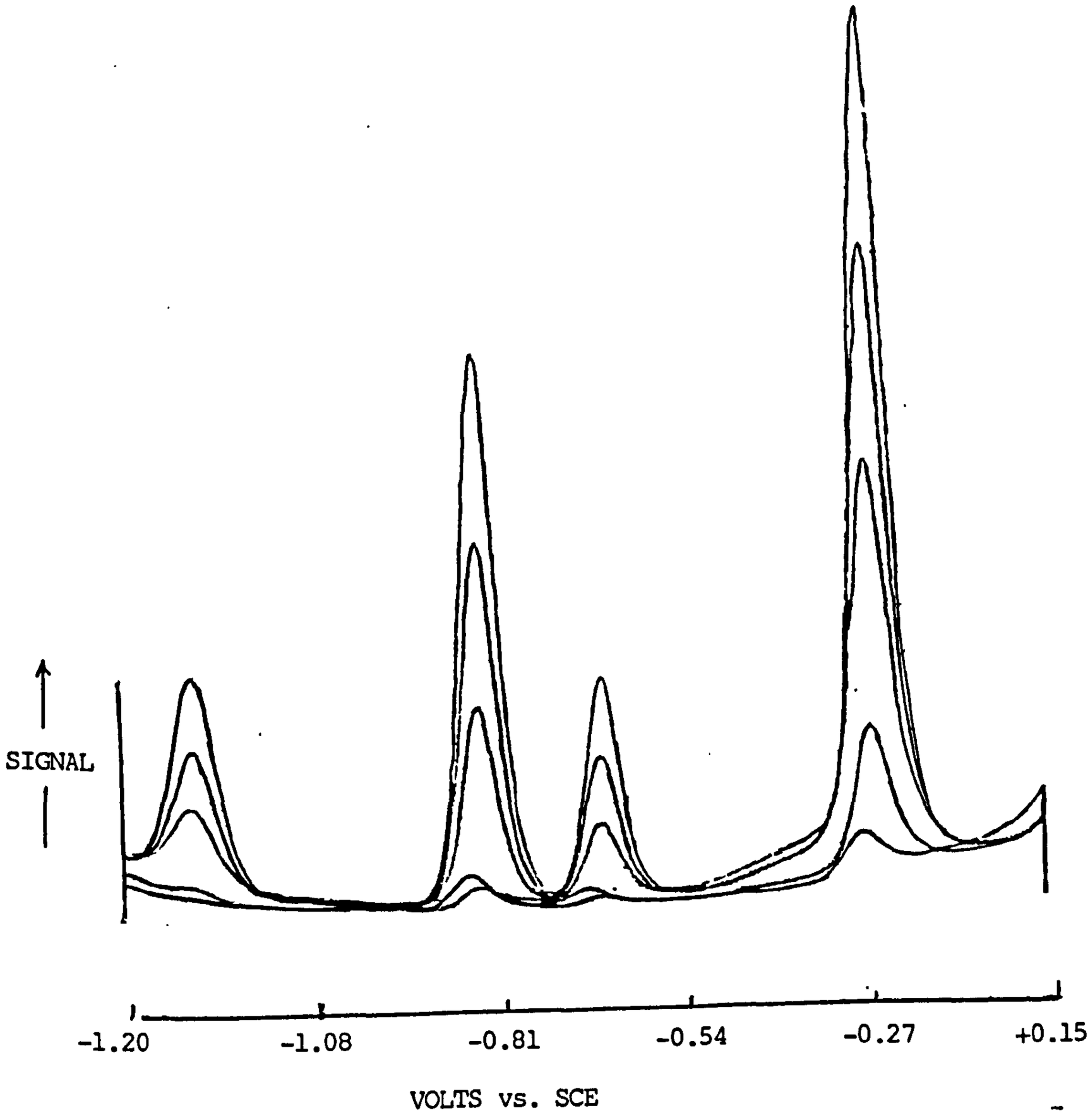


Table 26 : Preparation of pure acids by isothermal distillation

a) Molarity of original and prepared nitric acid

Time (days)	Molarity HNO_3	Molarity "DDW"
0	15.78	0.00
2	13.64	0.62
8	10.42	2.47
31	10.00	7.14

b) Molarity of original and prepared acetic acid

Time (days)	Molarity CH_3COOH	Molarity "DDW"
0	17.50	0.00
6	14.29	6.97
9	-	8.58
28	11.11	12.50

Table 27 : DPASV analysis of Bristol University tap water at pH 3 (0.2 M ammonium citrate)

(The sample was collected in the morning after resting in contact with the water pipes overnight)

	Concentration (ng/ml)			
	Zn	Cd	Pb	Cu
Tapwater	160.0	40.0	120.0	360.0

-

Table 27, tap water is contaminated with metals. Contamination was found to be a problem with deionised and single distilled water and some sources of DDW were not sufficiently pure. Cation exchange columns were used in an attempt to remove metal impurities from DDW.

Amberlite IRC-718 (Rohm and Haas - European Region) was used. The resin is based upon a macroreticular matrix, containing a chelating functionality, as a result of which it displays unusual selectivity for metals. Since it is a carboxylic resin, the hydrogen ion competes with metal ions for the carboxylic acid sites. In operation, this resin is best utilised in an alkali metal or ammonium ion form. Its capacity is pH dependent and while the resin can be used below pH 2, the maximum capacity for cations is above pH 4.

The resin was prepared as follows :- The sodium form of the resin (100 g) with a particle size range of 20 to 50 mesh, was slurry packed in a 2.5 cm i.d. column, to a height of 17.5 cm.

Amberlite IRC-718 resin was used for the purification of water and background electrolytes. The voltammograms in Figure 18 are of DDW before and after passage through the column, analysed at pH 3 (0.2 M ammonium citrate). The metal levels (in ng/ml) are given in Tables 28 and 29.

iii) Other methods for water purification

Millipore "Milli-Q" water system

This consists of a series of triple mixed bed cartridges, containing a Nuclear grade ion exchange resin mixed with a synthetic absorbant. The water is filtered to 0.22 μm. The feed used was tap water.

To obtain water of sufficient purity and quantity "Milli-Q" water was tested by DPASV (at pH 3). The resultant voltammogram is shown in Figure 19, indicating that the water was unsuitable.

Figure 18 : Differential pulse stripping curves (HMDE) of Zn, Cd, Pb and Cu in :-

a) Triply distilled water; b) DDW after passage through an Amberlite IRC-718 column; and c) DDW : 0.2 M ammonium citrate

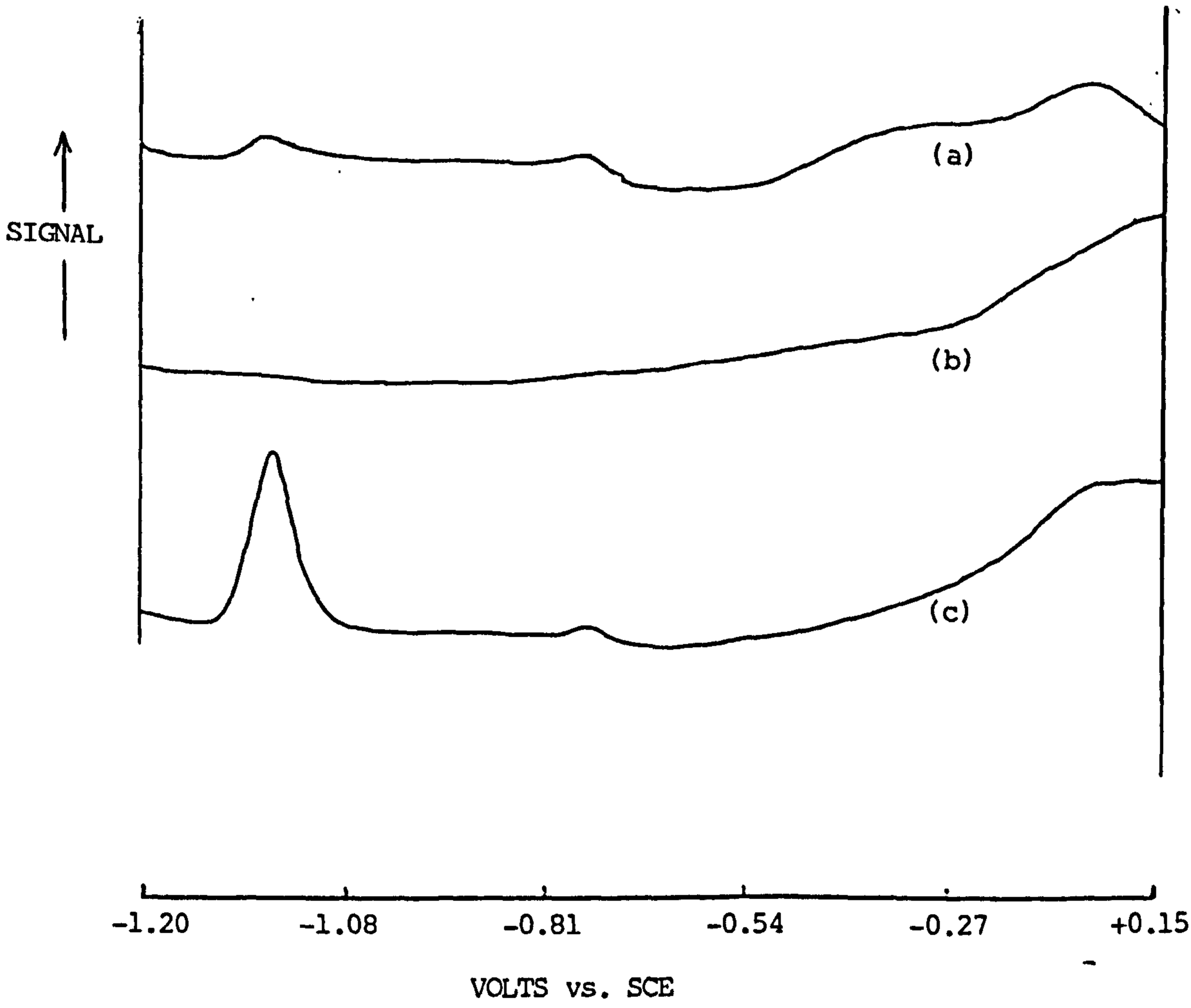


Figure 19 : Differential pulse stripping curve (HMDE) of Zn, Cd, Pb and Cu in Millipore 'Super Q' water : 0.2 M ammonium citrate

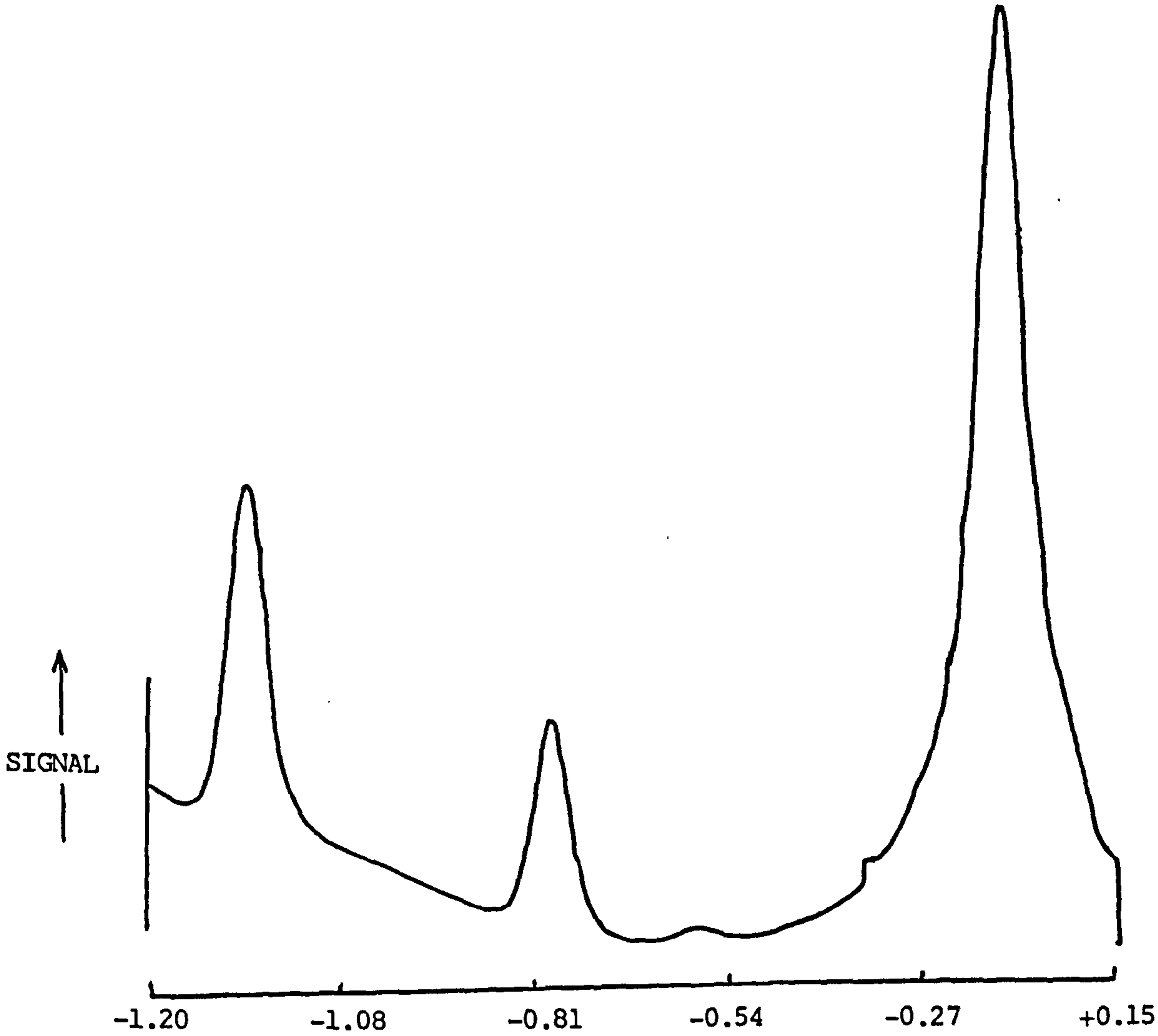


Table 28 : DPASV analysis of deionised water and DDW before and after passage through Amberlite IRC-718 (analysis at pH 3)

	Concentration ng/ml			
	Zn	Cd	Pb	Cu
DI water	116.0	2.0	4.0	154.0
DDW (before)	22.0	24.0	6.0	4.0
DDW (after)	1.0	1.0	4.0	N.D.

Table 29 : DPASV analysis of background electrolytes before passage through Amberlite IRC-718

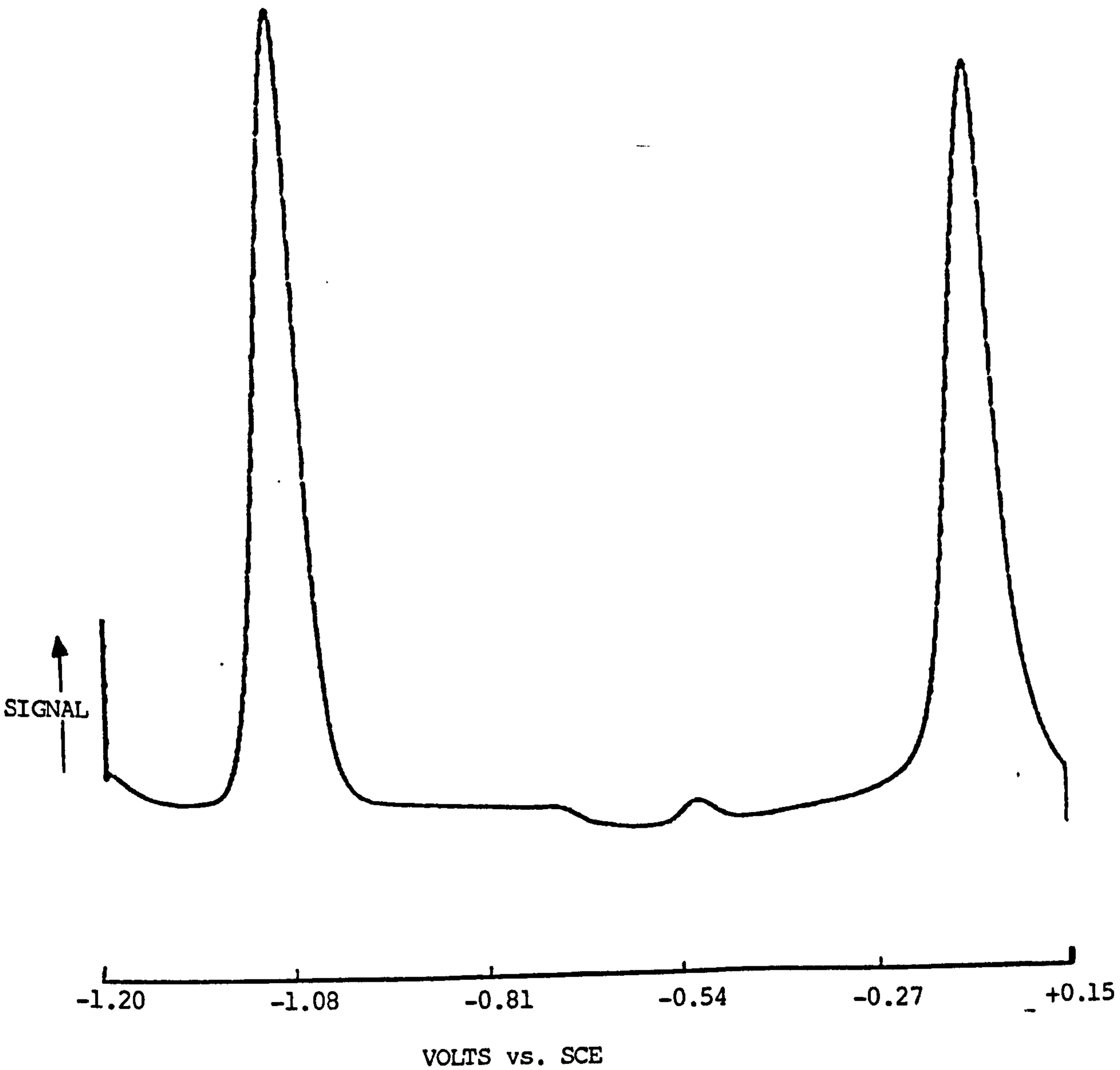
	Concentration ng/ml			
	Zn	Cd	Pb	Cu
Buffer pH 7	44.0	6.0	16.0	4.0
Buffer pH 5	52.0	4.0	14.0	6.0
Buffer pH 3	N.D.	2.0	6.0	N.D.

The metal levels were negligible (< 1 ng/ml) after purification.

Distillation/deionisation

Samples of double and triple distilled water and of deionised water were tested by DPASV (at pH 3). From the voltammograms in Figures 18, it can be seen that triply distilled water was of excellent purity, but was not available in sufficient quantities. Double distilled water was used since this was available in both sufficient quantity and purity. Single distilled and deionised waters (Figure 20) were unsuitable.

Figure 20 : Differential pulse stripping curve (HMDE) of Zn, Cd, Pb and Cu in 'deionised' water : 0.2 M ammonium citrate



iv) Other methods for purification of background electrolytes

Solvent extraction

The technique was carried out on pH 5 and pH 3 buffers.

pH 5 - 0.2 M sodium acetate. The solution was shaken twice with 100 ml of 100 mg/l dithizone in chloroform (purified by shaking with a pH 6.7 buffer of 1.3 g ammonium chloride and 4.4 ml (1 + 49 ml) ammonia in 500 ml water).

pH 3 - 0.2 M ammonium citrate. The solution was transferred to a large separating funnel, and 25 ml of an 8% w/v aqueous solution of ammonium pyrrolidine dithiocarbamate were added to the funnel. After gentle agitation for 10 minutes, 50 ml of chloroform were added. The solution was then shaken for 20 minutes. The chloroform layer was discarded. Ammonium pyrrolidine dithiocarbamate (5 ml of 1% w/v aqueous solution) was added to the aqueous layer. The extraction was repeated, the chloroform layer again being discarded. The aqueous electrolyte was washed by shaking twice with 50 ml volumes of chloroform. The final organic phase was discarded. Traces of chloroform were removed by bubbling nitrogen through the warm solution.

Both buffers were examined by DPASV. The resulting voltammograms are shown in Figures 21 and 22. These indicate that the background electrolytes are contaminated to such a degree that they are unsuitable for use with DPASV. The various stages of solvent extraction, apart from being time-consuming, also provide an increase in possible contamination sources.

Mercury cathode electrolysis

A 3 litre cell was designed (Figure 23), such that a pool of clean mercury encircled a low glass platform in the centre of the cell floor. A magnetic stirrer bar was located on this glass platform. The mercury

Figure 21 : Differential pulse stripping curve (HMDE) of Zn, Cd, Pb and Cu in 0.2 M sodium acetate (after repeated solvent extraction)

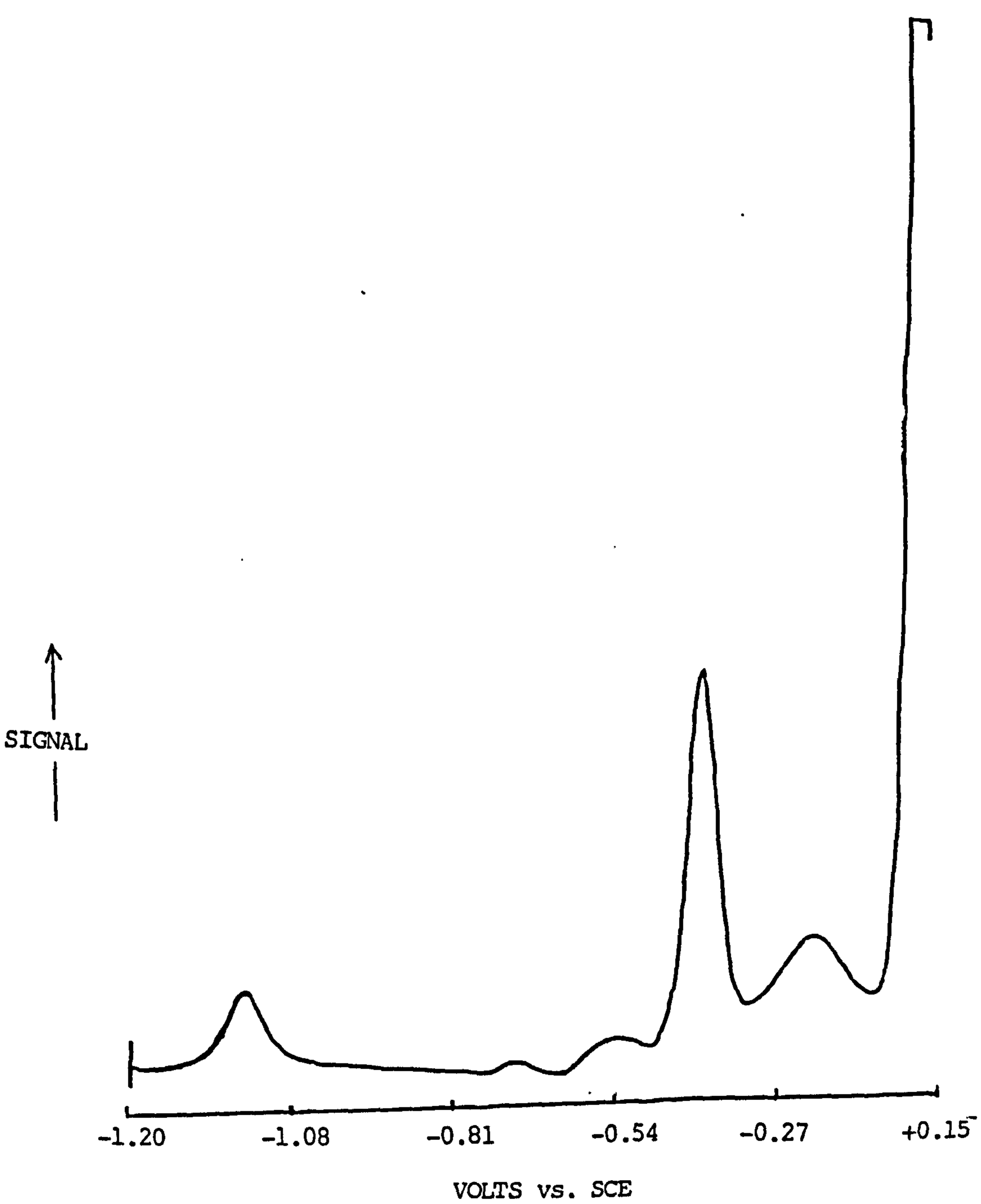


Figure 22 : Differential pulse stripping curve (HMDE) of Zn, Cd, Pb and Cu in 0.2 M ammonium citrate (after repeated solvent extraction)

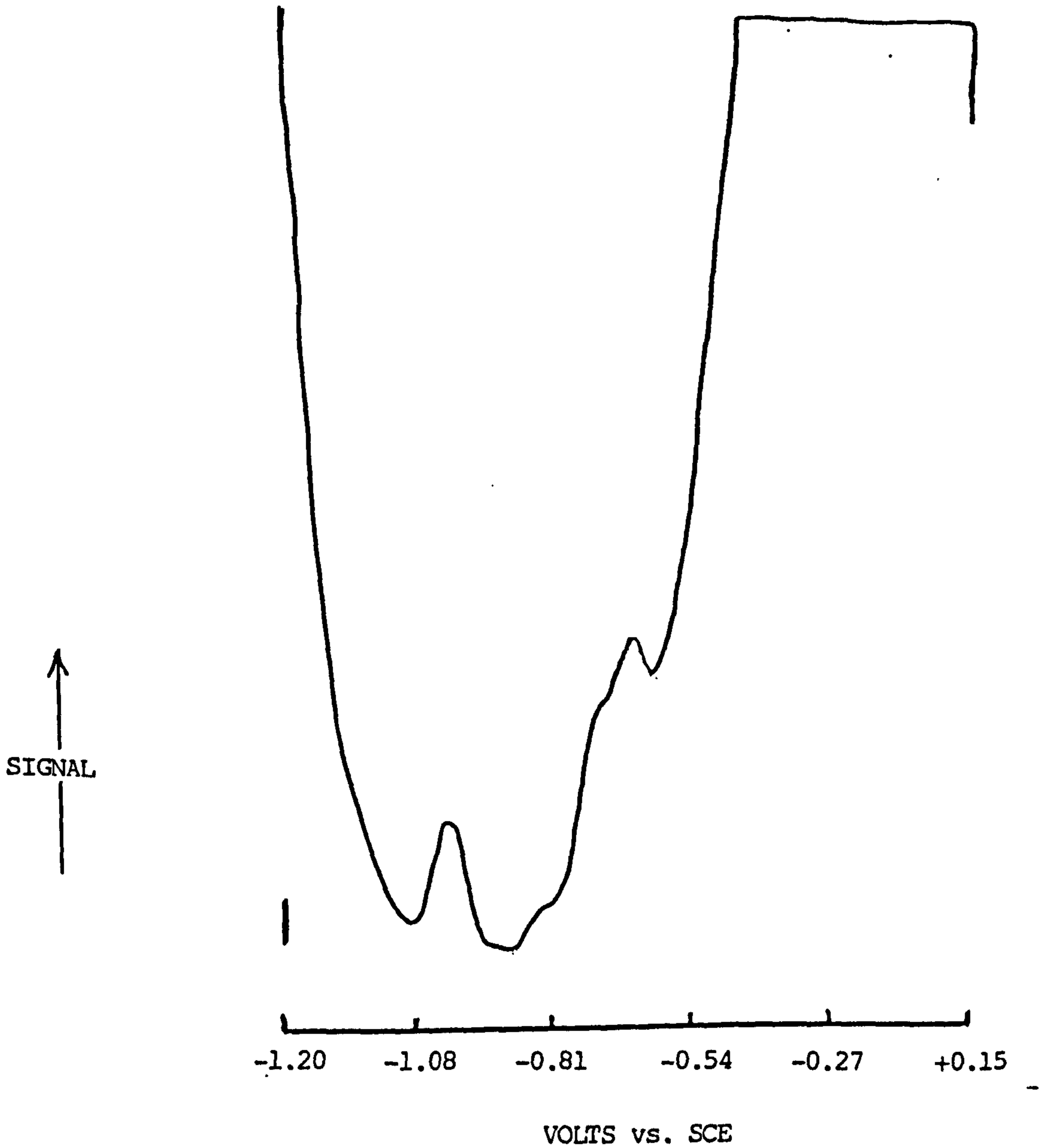
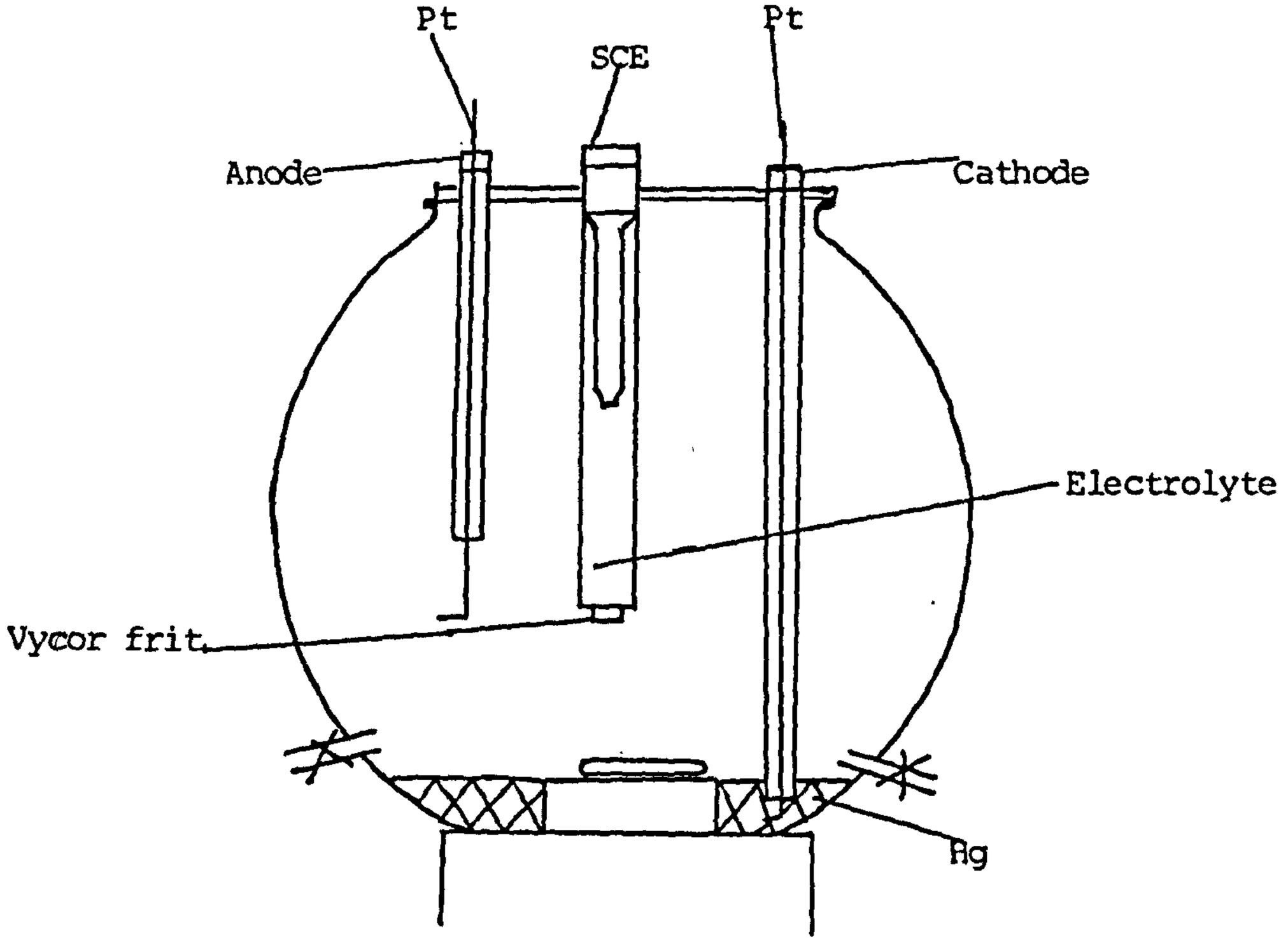


Figure 23 : Purification of background electrolytes by mercury cathode electrolysis



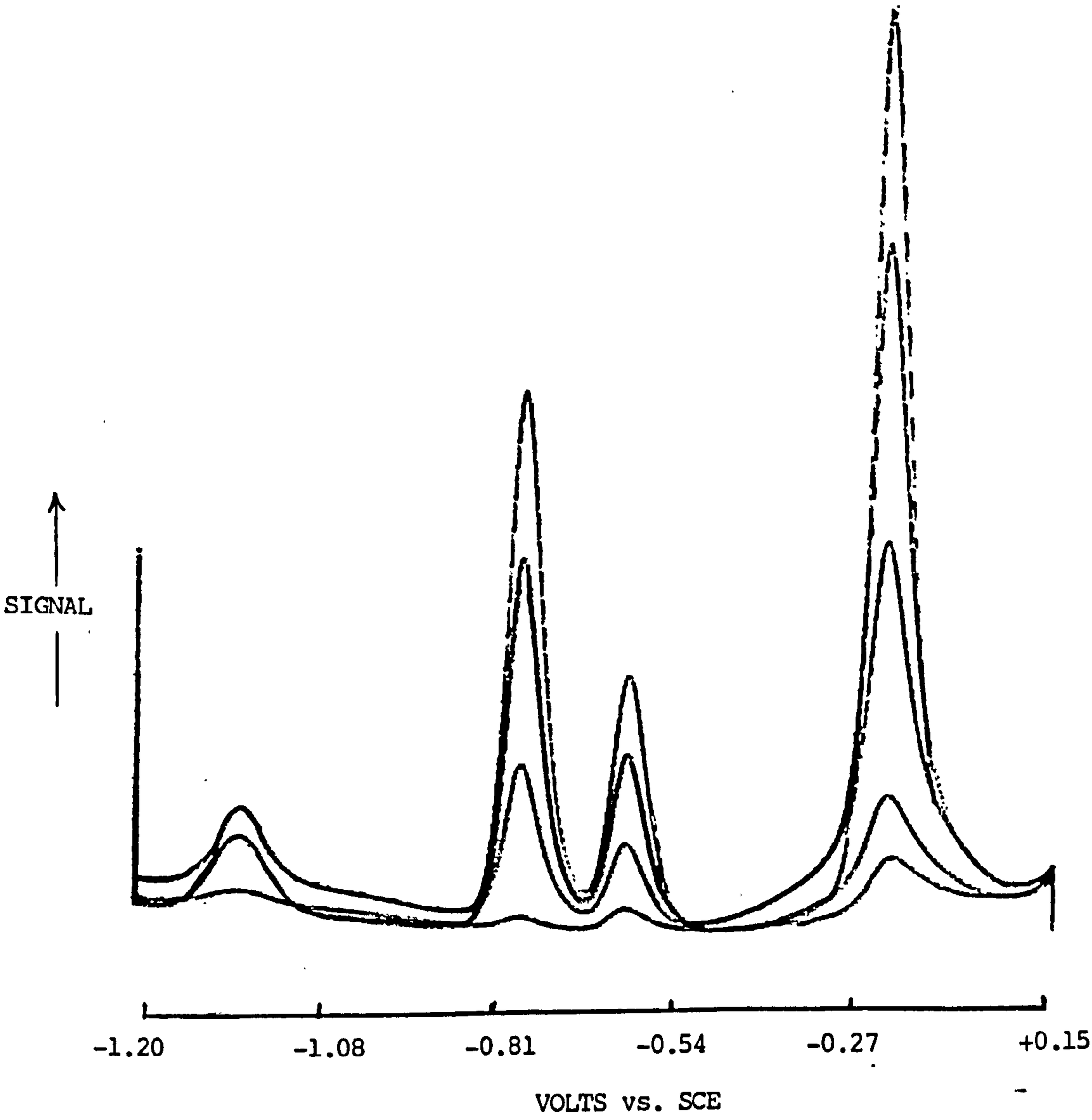
was made negative with respect to the buffer solution. The voltage of the mercury was gradually increased to -2 V, a voltage at which metals should be almost completely removed from the solution. A power operational amplifier potentiostat Regulated Power Supply Mp-1026 MP1 McKee-Pedersen Instruments (Danville, Calif. USA) was used. The power supply was maintained at +10 V and the output adjusted at 132 mA. The solution was stirred at a constant rate using a Gallenkamp Magnetic Stirrer and Hotplate Assembly. The stirrer speed was carefully maintained by turning the stirrer switch until it was just on, at higher rates the stirrer was dislodged from its platform and became lodged in the mercury pool.

Figure 24 is a voltammogram of ammonium citrate pH 3, after electrolysis. The metal levels in ng/ml are given in Table 30.

Table 30 : DPASV analysis of pH 3 background electrolyte (0.2 M ammonium citrate) after electrolysis

	Concentration ng/ml			
	Zn	Cd	Pb	Cu
pH 3 electrolyte	16	4	11	16

Figure 24 : Differential pulse stripping curve (HMDE) of Zn, Cd, Pb and Cu in 0.2 M ammonium citrate (after purification by mercury cathode electrolysis)



e) Site description

Two rainwater samples were obtained from the Botany Department, University of Bristol. The samples had been collected from deciduous woodland at Hallen (British Ordnance Survey grid reference ST 555 802) and Midger (ST 795 893). Hallen is a site of known metal contamination 2.8 km northeast (i.e. downwind) from the primary lead-zinc smelter at Avonmouth. Midger is 28 km northeast of Avonmouth, distant from any major roadway and considered to be relatively uncontaminated.

River water samples were obtained from the following sites :-

Afon Rheidol, Dyfed, west central Wales (SN 710 788);

Afon Ystwyth, Dyfed, West central Wales (SN 795 738)

River Caradon, Cornwall (SX 295 713)

Middlehill (ST 812 688)
St. Annes (ST 617 726)
Reybridge (ST 919 690)
Drewett's Mill (ST 784 662)
} River Avon

Luckett mine adit Cornwall (SX 385 736)

The Cwm Ystwyth mine was one of the largest mines in Wales, yielding both lead and zinc ores until it was closed in 1893 (274). The Cwm Rheidol was finally abandoned around 1922 (275). Wind and water erosion of the waste material has led to continued river pollution. At the Cwm Rheidol mine unauthorised exploitation by mineral prospectors breached a flooded adit at the top of the dumps, about 10 years ago, and since then large quantities of metal have been transported from the mine into the river. Attempts have been made to ameliorate the effects of the leachate on the river by the installation of a limestone trap to increase the pH of the water before it enters the river.

At both the Cwm Rheidol and Cwm Ystwyth mines the red deposits of iron oxide are present.

The Caradon group included six mines, of which South Caradon was the richest. The mines were not active until around 1830, but for the following 50 years, the group became one of the most important copper-producing areas of Cornwall. The lode-stuff and waste produced was concentrated by various methods of gravity separation. The South Caradon mine was finally abandoned in 1890. Mine waste still remains (276,277).

The Bristol Avon is a large river rising on oolite. The By Brook and River Frome also flow on oolite, but there are small outcrops of coal measures and sandstones in the catchments of the Avon, Frome and By Brook. The Kennet and Avon canal joins the Avon near Bradford and the river is canalised below this, with complex wharf systems in Bath and Bristol. The main River Avon passes onto clay a few miles above Malmesbury and a clay tributary also enters above the town. A survey carried out after 1980 indicated that By Brook had improved in diversity and cleanliness, but the pollution above Malmesbury was more noticeable, as was the pollution on the main River Avon (278). The St. Annes site is near the centre of Bristol; Middlehill is in the vicinity of Box, near a sewage works; Reybridge is near Lacock and Drewett'sMill is near Bathampton.

The main products of the mines around Luckett were copper, arsenic and pyrite. Four mines were situated around Luckett, the last of these was finally abandoned in 1952. As well as copper, arsenic, pyrite and tin, traces of gold, silver and lead were found at these mines, and mining for lead and silver was carried out in 1854 at the West Devon Consols with the sharp bend between Luckett and Latchley (276).

The sampling sites are indicated in Figure 25.

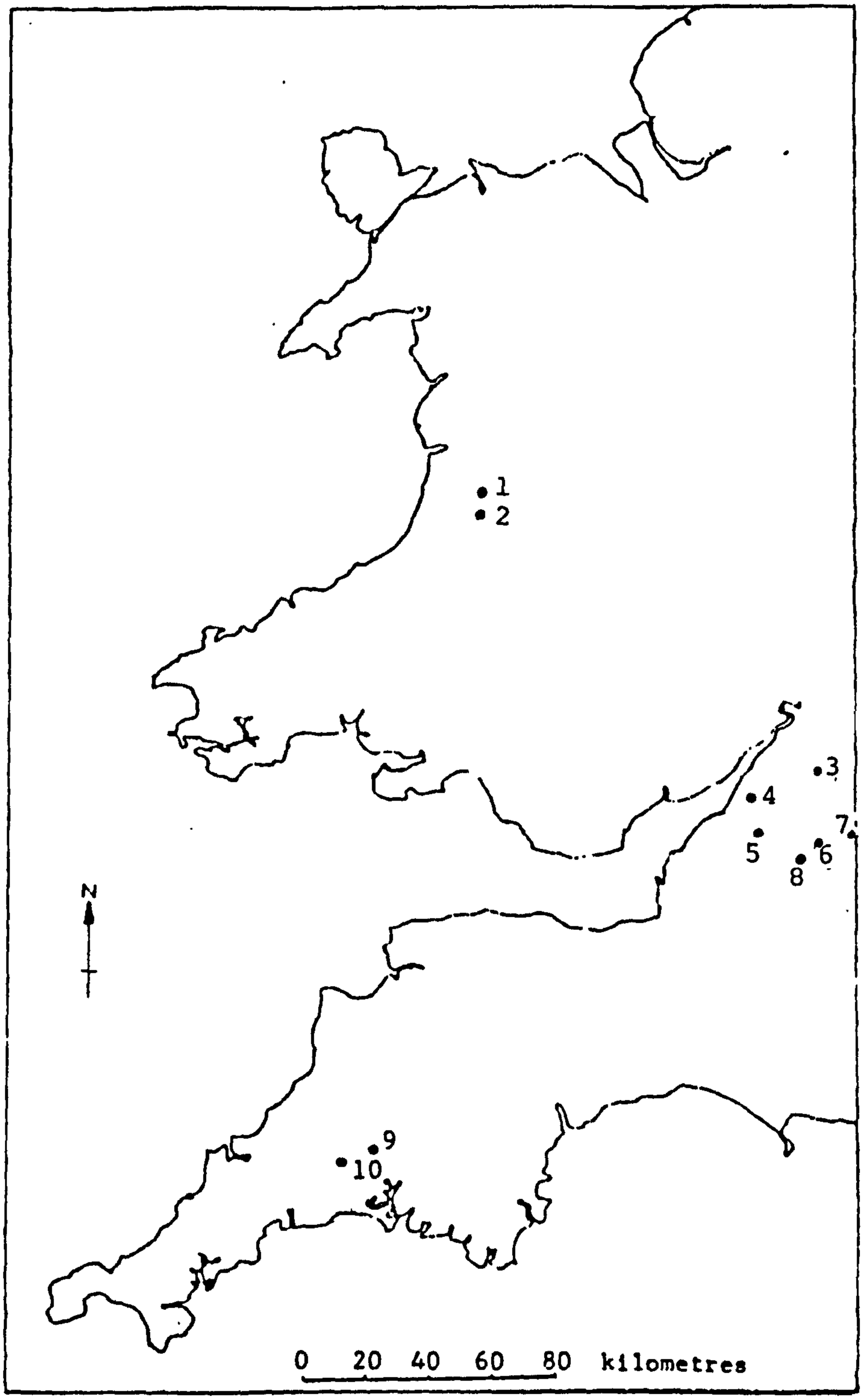


Figure 25 : Location of water sampling sites

KEY :
1. R. Rheidol
2. R. Ystwyth
3. Midger Wood
4. Hallen Wood
5. St. Annes Boardmill
6. Middlehill
7. Reybridge
8. Drewett's Mill
9. Luckett
10. Caradon

f) Sample collection

The samples were collected in polypropylene vessels which had been washed in 2% v/v nitric acid for 24 hours and then rinsed twice with DDW. The vessels were rinsed 3 times on site with the sample water.

The samples were analysed by DPASV as soon as possible, usually within 24 hours, where this was not possible, the samples were stored at $4^{o}C$.

g) Sample treatment, analysis and results

Cwm Rheidol and Cwm Ystwyth samples

Five samples were collected along the length of a mine adit as it progressed downwards, across the mine dumps at Cwm Rheidol (samples R_5 - at the top of the dump, to R_1 - at the bottom). Two samples were collected in the vicinity of the carbonate trap, one before entry (RB) and one immediately after passage through the trap (RA)

At Cwm Ystwyth, a sample was taken from a mine adit and from the river nearby (Y_1 and Y_2 respectively). Two other samples were collected from the River Ystwyth (Y_3 and Y_4).

Aliquots of samples from the Rheidol and Ystwyth were left unfiltered and analysed directly at a pH of 3 (0.2 M ammonium citrate) by DPASV. The zinc levels were very high and were determined by FAAS (samples were then filtered through Whatman 541 filter paper to prevent blockage of the nebuliser). The results are listed in Table 31.

An attempt to fractionate the metal species by size was carried out on one sample from each mine site, by using membrane filters of 5 μm, 0.45 μm and 0.1 μm pore size. The fractions were buffered at pH 3 (0.2 M ammonium citrate) and stored in polythene bottles. Analysis was carried out by FAAS. The results are given in Table 32.

Table 31 : Ystwyth (Y) and Rheidol (R) water samples unfiltered and determined by DPASV at pH 3 (0.2 M ammonium citrate). *Analysis by FAAS (*results in µg/ml)

	Concentration ng/ml			
	Zn*	Cd	Pb	Cu
R_5 (TOP)	10.1	24.0	640.0	28.0
R_4	10.4	28.0	632.0	38.0
R_3	10.6	32.0	672.0	36.0
R_2	11.4	10.0	880.0	32.0
R_1 (BOTTOM)	29.9	64.0	520.0	68.0
Y_1 (adit)	17.4	13.0	48.0	N.D.
Y_2	0.9	2.0	44.0	2.0

Table 32 : Ystwyth (Y) and Rheidol (R) samples filtered through 5, 0.45 and 0.1 µm, buffered at pH 3 (0.2 M ammonium citrate) and determined by FAAS

		Concentration µg/ml
Sample	Size fraction (µm)	Zn
Y_1	5	16.7
Y_1	0.45	7.5
Y_1	0.1	14.1
R_5	5	10.2
R_5	0.45	7.5
R_5	0.1	6.6

Fractionation of the metal species by alteration of the pH was attempted initially on one of the Ystwyth river samples. The sample was filtered through a 5 µm membrane filter and then aliquots were determined at pH 7 (0.2 M ammonium acetate), pH 5 (0.2 M sodium acetate) and pH 3 (0.2 M ammonium citrate). A "total" (acid digest) metal determination was carried out; 50 ml of the water sample was heated with 5 ml of concentrated nitric acid and the mixture concentrated to a volume of approximately 2 ml. The concentrate was diluted with DDW and brought to a pH of 3 with ammonia solution, before being diluted to a final volume of 50 ml. Analysis was carried out at pH 3 (0.2 M ammonium citrate) by DPASV. The results are given in Table 33 and a voltammogram of Y_3 acid digest <5.0 µm is given in Figure 26.

Fractionation using both size and change in pH was applied to one sample from each of the mine sites. Filtration was carried out using 5 µm, 0.45 µm and 0.1 µm membrane filters and analysis was carried out at pH 7, 5 and 3 as described previously. The results are given in Table 34.

The Rheidol samples collected before and after the carbonate trap were left unfiltered and analysed at pH 3 (0,2 M ammonium citrate) by DPASV (Table 35).

River Caradon sample

The sample had already been acidified with nitric acid. Analysis was carried out on an unfiltered aliquot at a pH of 3 (0.2 M ammonium citrate) by DPASV, Table 36 (Figure 27).

Table 33 : Ystwyth river sample, after filtration through 5 µm, at pH 7, 5 and 3 (ammonium acetate, sodium acetate and ammonium citrate respectively). Anal ysis by DPASV

(Y_3) pH	Zn concentration µg/ml
7	2.4
5	1.6
3	4.4
Acid digest	26.8

Table 34 : Ystwyth (Y) and Rheidol (R) water samples fractionated by size and pH. Analysis by DPASV

Site	Size fraction (µm)	Zn concentration µg/ml pH 7	pH 5	pH 3	(acid digest) pH 3
R_1	5	8.8	12.8	28.0	30.0
	0.45	8.4	13.2	17.6	19.2
	0.1	8.0	14.0	10.8	15.2
Y_4	5	26.6	15.3 26.6*	32.6	33.0
	0.45	23.6	14.4 16.8*	22.4	28.0
	0.1	20.0	14.4 14.4*	19.9	20.0

* ammonium citrate at pH 5 used as background electrolyte

Figure 26 : Differential pulse stripping curve (HMDE) for Zn, Cd, Pb and Cu in Ystwyth (Y_3) < 5 µm sample : 0.2 M ammonium citrate

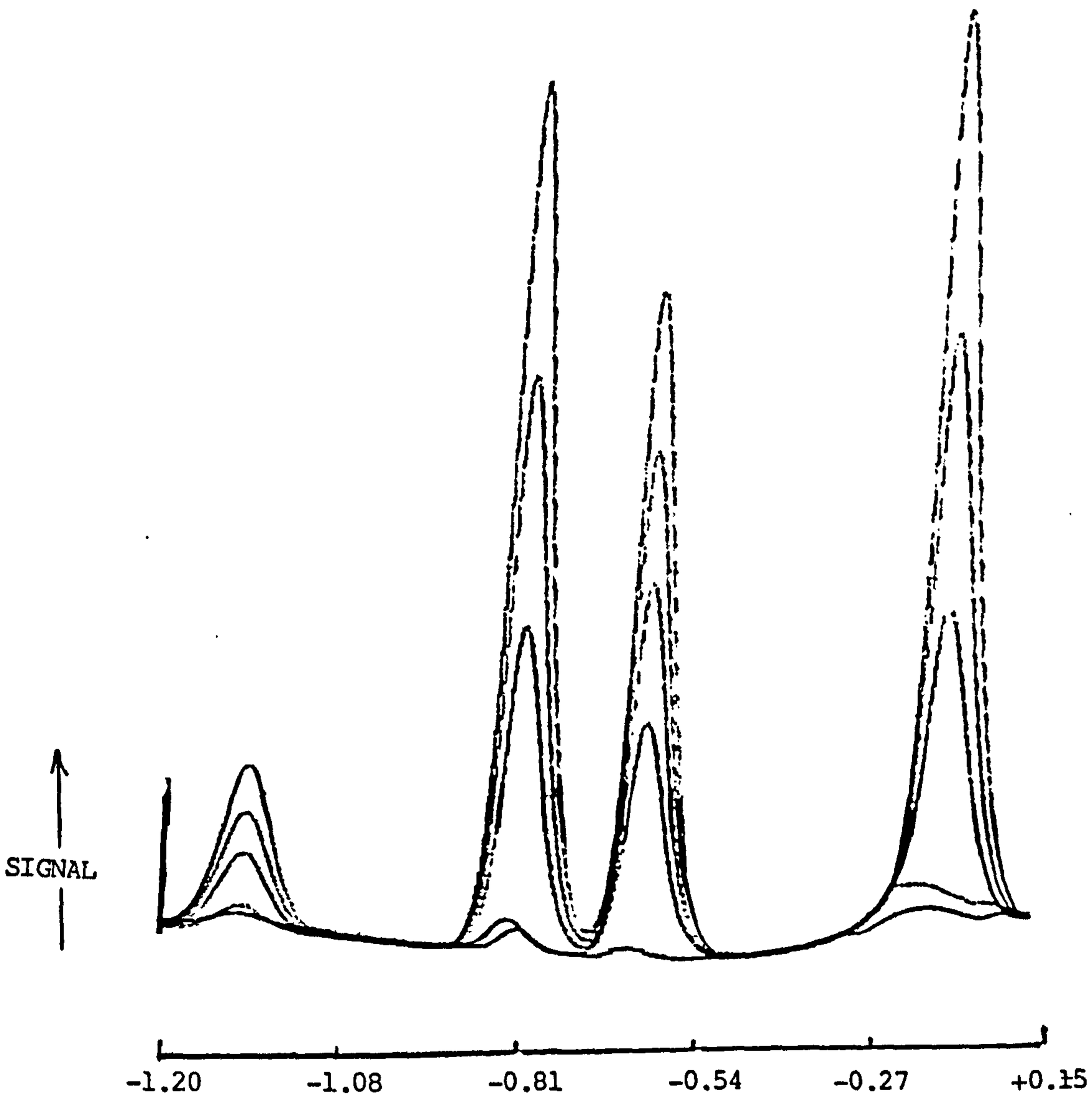


Table 35 : Rheidol adit before (RB) and after (RA) passage through a carbonate trap, (unfiltered, and analysed by DPASV at pH 3 0.2 M ammonium citrate)

	Concentration (ng/ml)			
Sample	Zn	Cd	Pb	Cu
RB	21 200.	166.	568	81
RA	52	N.D.	62	4

Table 36 : River Caradon acidified water sample, unfiltered, analysed by DPASV at pH 3 (0.2 M ammonium citrate)

	Concentration (ng/ml)			
	Zn	Cd	Pb	Cu
Caradon	666	N.D.	N.D.	1866

River Avon samples : St. Annes, Middlehill, Drewett's Mill and Reybridge

The samples were left unfiltered and analysed directly by DPASV at pHs 7 and 3 (0.2 M ammonium acetate and ammonium citrate respectively). The results are shown in Table 37.

Rainwater samples

The samples were filtered through 0.45 µm membrane filters of known weight. The residues were dried at 50°C, weighed and then heated with 10 ml of concentrated nitric acid, and concentrated to approximately 2 ml. The concentrate was diluted with DDW and brought to a pH of 3 with ammonia solution, before being diluted to a volume of 25 ml. Aliquots of the

filtrate were examined at a pH of 3 (0.2 M ammonium citrate) by DPASV. A "total" metal acid digest of the filtrate was also carried out, as described previously. The results are listed in Table 38.

Mine drainage, Luckett

An aliquot of the sample was filtered through a 0.45 µm membrane filter. Analysis was carried out by FAAS on both the unfiltered and filtered aliquots. The results are listed in Table 39.

Table 37 : River Avon Samples : DPASV analyses at pH 7 and 3, on unfiltered samples

		Concentration (ng/ml)			
Site	pH	Zn	Cd	Pb	Cu
Reybridge	7	10.0	4.0	4.0	N.D.
	3	66.0	4.0	4.0	N.D.
Middlehill	7	12.0	3.0	1.0	2.0
	3	N.D.*	6.0	2.0	N.D.

* interference at pH 3 for Zn

The Drewett's Mill and St. Anne samples were found to contain metal levels similar to those of the background electrolytes.

Figure 27 : Differential stripping curve (HMDE) of Zn, Cd, Pb and Cu in 6% v/v Caradon water sample : 0.2 M ammonium citrate

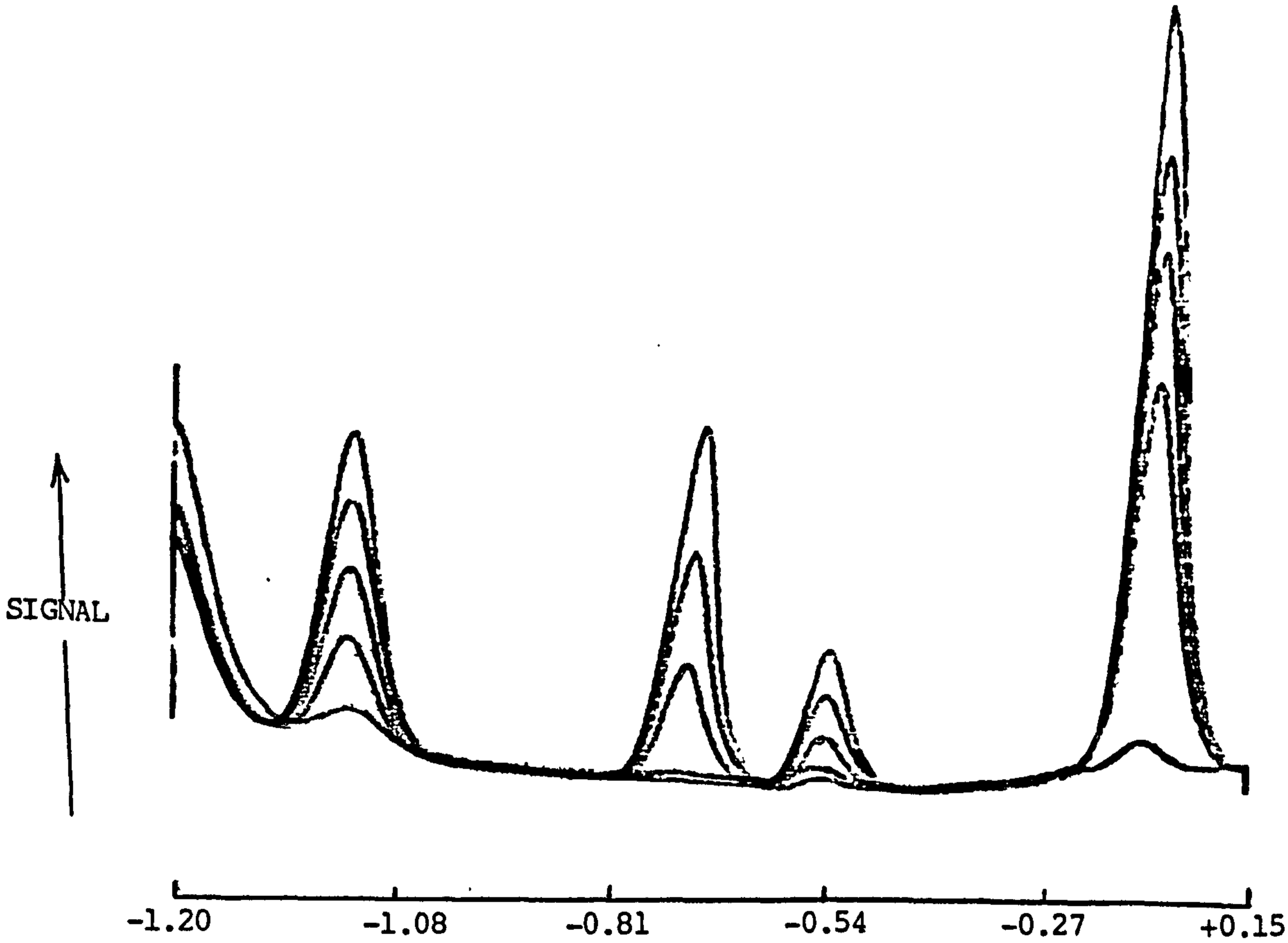


Table 38 : Rainwater samples from Hallen and Midger woods, filtered at 0.45 µm and analysed at pH 3 by DPASV. *Residue values in µg/g

Sample	Size fraction (µm)	Concentration (ng/ml) Zn	Cd	Pb	Cu
Hallen pH 3	< 0.45	100.0	N.D.	52.0	42.0
Hallen AD*	< 0.45	354.0	6.0	70.0	68.0
Hallen AD*	> 0.45	1697.0	109.5	1751.8	365.0
TOTAL ACID DIGEST* (< 0.45 + > 0.45)		2051.0	115.5	1821.8	433.0
Midger pH 3	< 0.45	53.0	N.D.	16.0	10.0
Midger AD*	< 0.45	80.0	4.0	22.5	10.5
Midger AD*	> 0.45	1406.0	41.6	1580.7	133.0
TOTAL ACID DIGEST* (< 0.45 + > 0.45)		1486.0	45.6	1603.2	143.5

Table 39 : Mine drain, Luckett, filtered at 0.45 µm and unfiltered. Analysis by FAAS

Sample	Size fraction (µm)	Concentration (µg/ml) Zn	Cu
1	< 0.45	8.8	17.2
2	< 0.45	7.0	17.5
Mean	< 0.45	7.9	17.4
1	unfiltered	9.2	14.4
2	unfiltered	9.6	15.7
Mean	unfiltered	9.4	15.0

h) Apparatus and instrumental parameters

Analysis by DPASV of heavy metals in prepared sample solutions

Apparatus :-

Model 174A Polarograph Analyser, Princeton Applied Research Corporation (PARC)

Model 315A Automated Electroanalysis Controller, PARC

Model 303 Static Mercury Drop Electrode, PARC

Model 305 Stirrer, PARC

Model 7040A X-Y Recorder, Hewlett Packard.

Method :-

The DPASV analyses were performed on 5 ml aliquots of the prepared sample diluted with 5 ml of the required electrolyte buffer, in a polarographic cell. In all cases, calibration was carried out by the method of standard additions. Each addition was of 20 µl of a new standard metal solution (10 µg/ml), prepared by dilution of stock metal solutions. The metal content of the sample was obtained by extrapolation and the concentration calculated.

Instrumental Conditions

Model 303 :

Electrode	HMDE (medium size)

Model 315A :

Conditioning Potential	0.15 V vs. SCE
Initial Potential	-1.2 V vs. SCE
Final Potential	0.15 V vs. SCE
Purge Time	10 min.
Conditioning Time	0 min
Equilibration Time	30 s
Deposition Time	120 s

Model 174A :

Scan Rate	5 mV/s
Scan Direction	"+"
Modulation Amplitude	25 mV
Current Range	10 μA
Drop Time	0.5 s
Display Direction	"-"
Low Pass Filter	"OFF"
Mode	Differential Pulse
Initial Potential	0.0
Potential Scan Rate	1.5 V

A diagram of the Static Mercury Drop Electrode is given in Figure 28.

Analysis by FAAS of heavy metals in prepared sample solutions

Apparatus :-

An Instrumental Laboratory Ltd. atomic absorption spectrophotometer model 151 with background correction facilities.

Method :

The analytical method involved the use of standard solutions for a direct comparison measurement to the unknown sample. The standards were prepared by dilution of the 1000 μg/ml stock solutions to give a range of standards suitable for the calibration plots. Since the working curve can change from day to day due to small variations in lamp and flame conditions, the instruments had to be recalibrated for each batch of samples. The analyte concentrations for solid samples were determined by the equation :-

$$Y = \frac{x\ k\ l}{z}$$

where Y = concentration in μg/g of metal in the sample

x = concentration in μg/g of metal determined from the calibration graph (actually in solution)

k = final volume (ml) of analyte solution

l = dilution factor (if any)

z = weight of sample (g) (if applicable)

Instrumental conditions :-

	Zn	Cd	Cu	Pb
Slit width (μm)	320	320	320	320
Wavelength (nm)	213.9	228.8	324.7	217.0
Lamp current (mA)				
Scale Expand (usual)	5	5	5	5
Curve correct	0	0	0	0
Integration Time (s)	1	1	1	1
Flame	---------- air/acetylene ----------			
Range of Standards (μg/ml) (usual)	0.5 to 2	0.5 to 2	0.5 to 10	0.5 to 10

Calibration graphs are given in Figures 29 and 30.

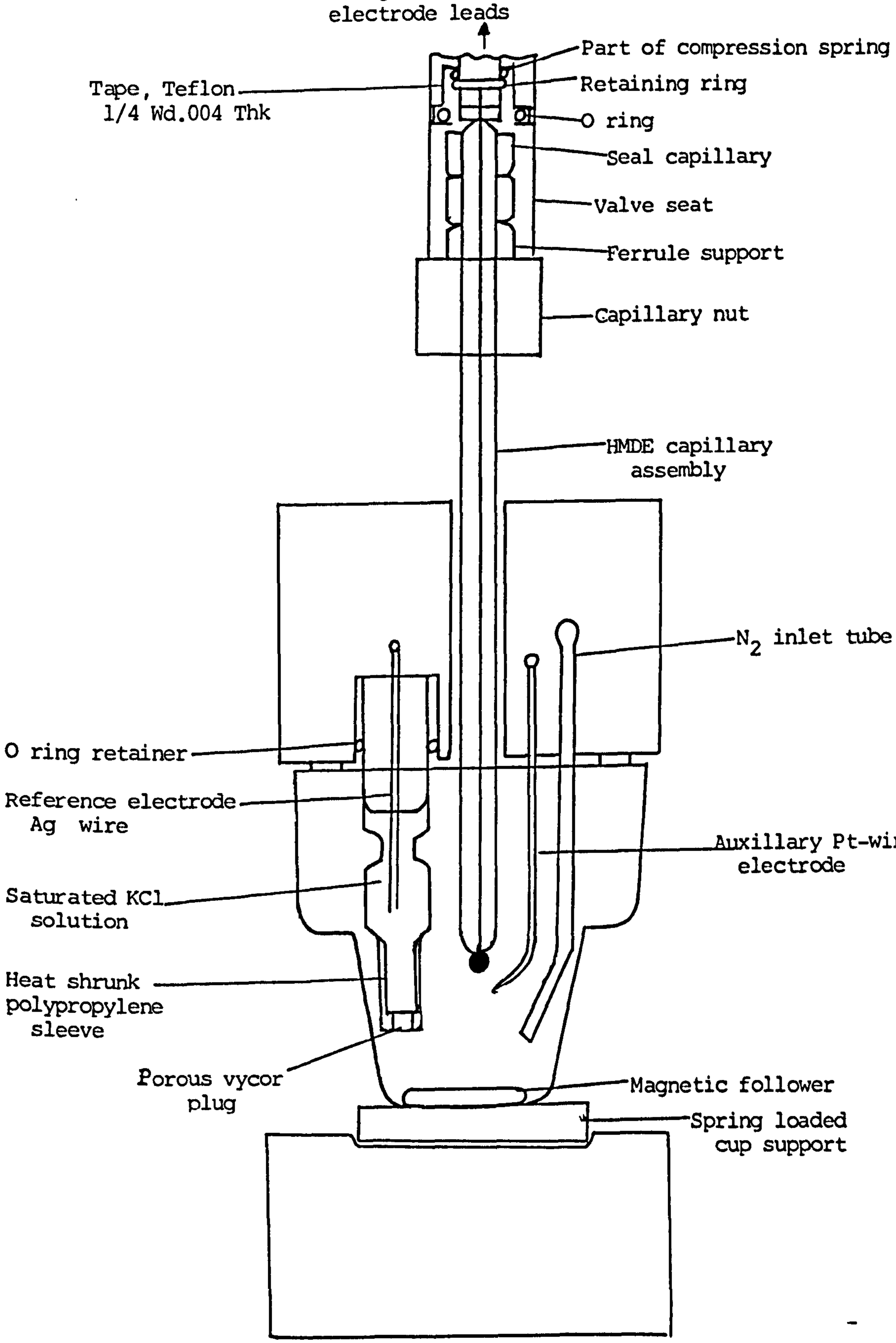
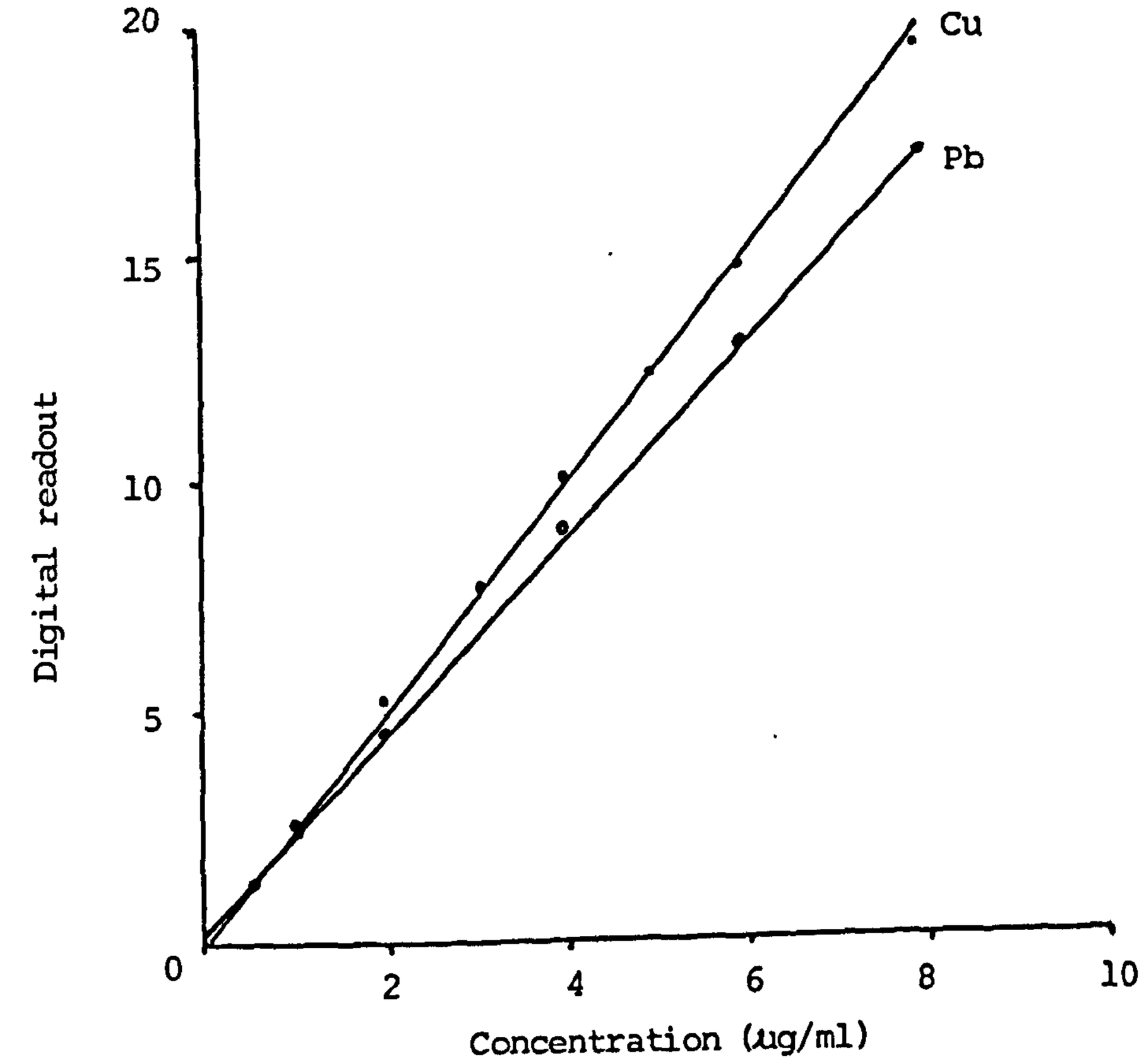


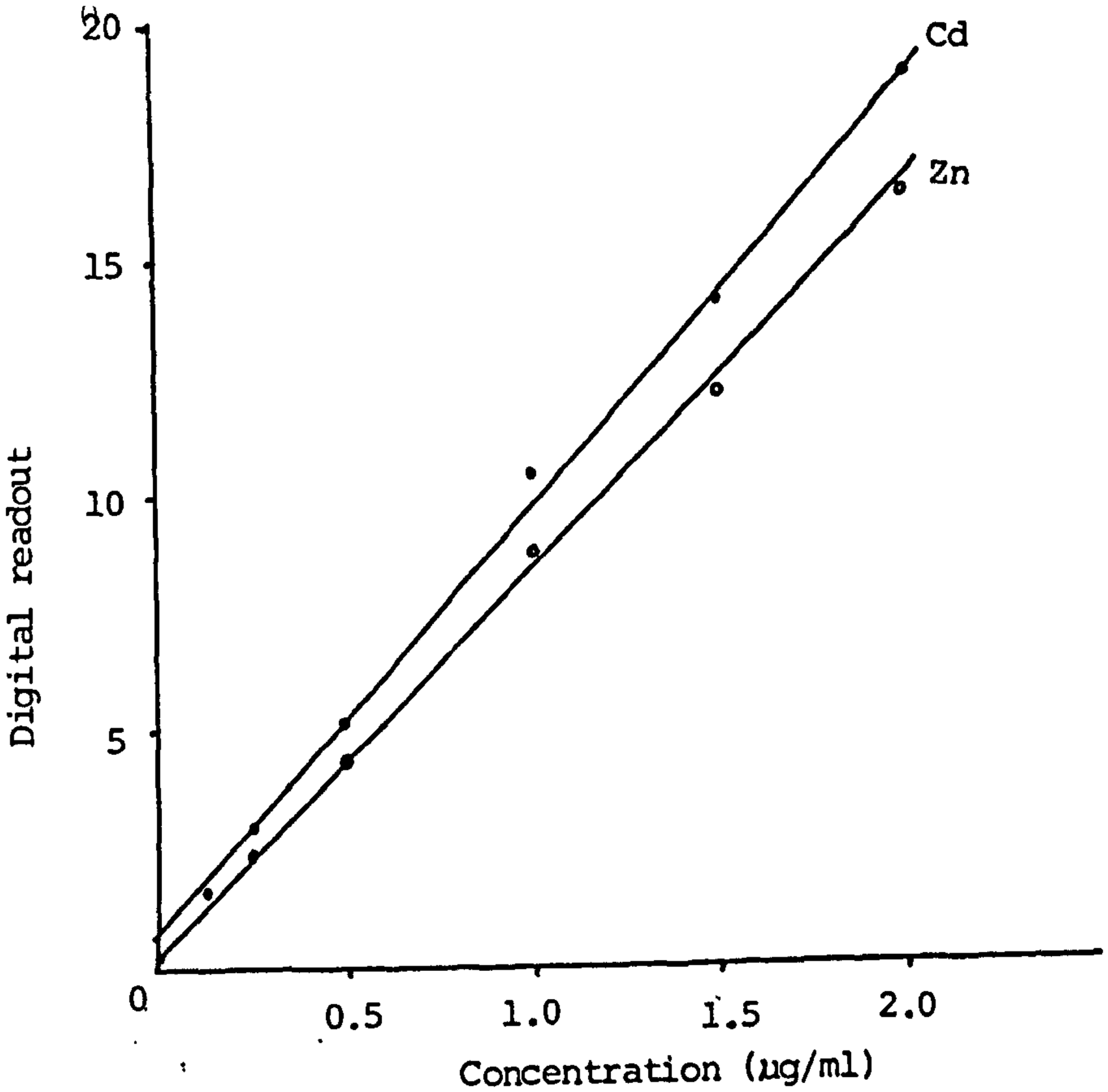
Figure 28 : PARC Model 303 Static Mercury Drop Electrode

Figure 29 : Calibration Graphs for Cu and Pb by FAAS (ILL 151)



	Cu	Pb
Gradient	2.42	2.12
Error on gradient	0.03	0.02
Correlation	0.996	0.9997
Intercept on Y axis	0.14	0.21
Maximum and minimum values of intercept	0.22 & 0.06	0.27 & 0.14
Standard deviation (x)	2.77	2.77
Standard deviation (y)	6.71	5.88
average (x)	3.11	3.11
average (y)	6.81	6.81

Figure 30 : Calibration Graphs for Cd and Zn by FAAS (ILL 151)



	Cd	Zn
Gradient	9.18	8.09
Error on gradient	0.19	0.10
Correlation	0.9987	0.9995
Intercept on Y axis	0.61	0.22
Maximum and minimum values of intercept	0.77 & 0.43	0.30 & 0.15
Standard deviation (x)	0.68	0.7
Standard deviation (y)	6.28	5.67
average (x)	0.90	0.77
average (y)	8.83	6.44

SECTION 2 : SEDIMENTS : METAL SPECIATION STUDIES

2.1 Experimental Preparations

a) Reagents

b) Site description

c) Sample collection

d) Sample treatment, Analysis and Results

e) Apparatus and Instrumental Parameters

SECTION 2 : SEDIMENTS : METAL SPECIATION AND FRACTIONATION STUDIES

2.1 Experimental Preparation

a) Reagents

AnalaR and AristaR reagents were used :-

AnalaR and AristaR nitric and acetic acid. AnalaR cadmium, copper, zinc and lead nitrates, ammonium acetate, citric acid, 0.91 ammonia solution, and sodium acetate.

b) Site description

Sediment samples were collected from Mineries Pool (ST 546 508) near Priddy in the Mendip Hills and the River Caradon (SX 295 713), Cornwall.

The Mendip Hills, in Somerset, are a plateau-like range of hills rising steeply from the surrounding low-lying river valleys. The region consists predominantly of Carboniferous limestone, with caves, swallets and underground streams. Lead mining has occurred in the Mendip Hills for centuries. Roman remains imply that mining may have occurred at Priddy. The Roman smelting process left a high proportion of lead in the slag, which was often resmelted by later miners. During the Middle Ages the washing and smelting was carried out at 4 main sites, one of which was at Priddy Minery. Mendip lead mining ended in the nineteenth century. Contaminated waste, however, remains a problem (279,280).

Four sediment samples from Garston Docks, River Mersey (SJ 395 843) were obtained from the Associated British Ports, Research Station, Southall.

The Mersey is one of the largest English rivers, lined with docks, oil refineries and large industrial enterprises. Tidal flow is

constricted by the narrow strait between the sandstone hills of Liverpool and Wallasey, thus assisting channel scour (281).

Slag samples were obtained from a site of archaeological interest, by the Geology Department, University of Bristol. Roman slag at Lawrence Weston (ST 545 784) had been mixed with twentieth century slag from Avonmouth (ST 525 790).

c) Sample collection

The samples were collected in polyethylene containers. The Mersey samples were supplied in glass bottles.

The sampling sites are indicated in Figure 31.

d) Sample treatment, analysis and results

Caradon and Mineries Pool Samples

The analyses were performed on the -80 mesh portion of the samples. The Mineries Pool sediment (2 g) was mechanically shaken (Mk. V Orbital Shaker, L.H. Engineering Co. Ltd., Speed 8) for 3 days with 200 ml of pH 7 (0.2 M ammonium acetate) background electrolyte. A further 2 g of this sediment was shaken with 200 ml of pH 5 (0.2 M sodium acetate) background electrolyte and a final 2 g were shaken with pH 3 (0.2 M ammonium citrate) background electrolyte. After shaking, the solutions were sequentially filtered through 5 μm, 0.45 μm and 0.1 μm membrane filters, aliquots of the filtrates being removed for acid digestion. This fractionation of the sediment on the basis of pH change and size is outlined in Figure 32. The Caradon sediment was also subjected to this scheme. The results are given in Tables 40 and 41 and a typical voltammogram of the Mineries Pool sediment < 0.45 μm, pH 7, is given in Figure 33.

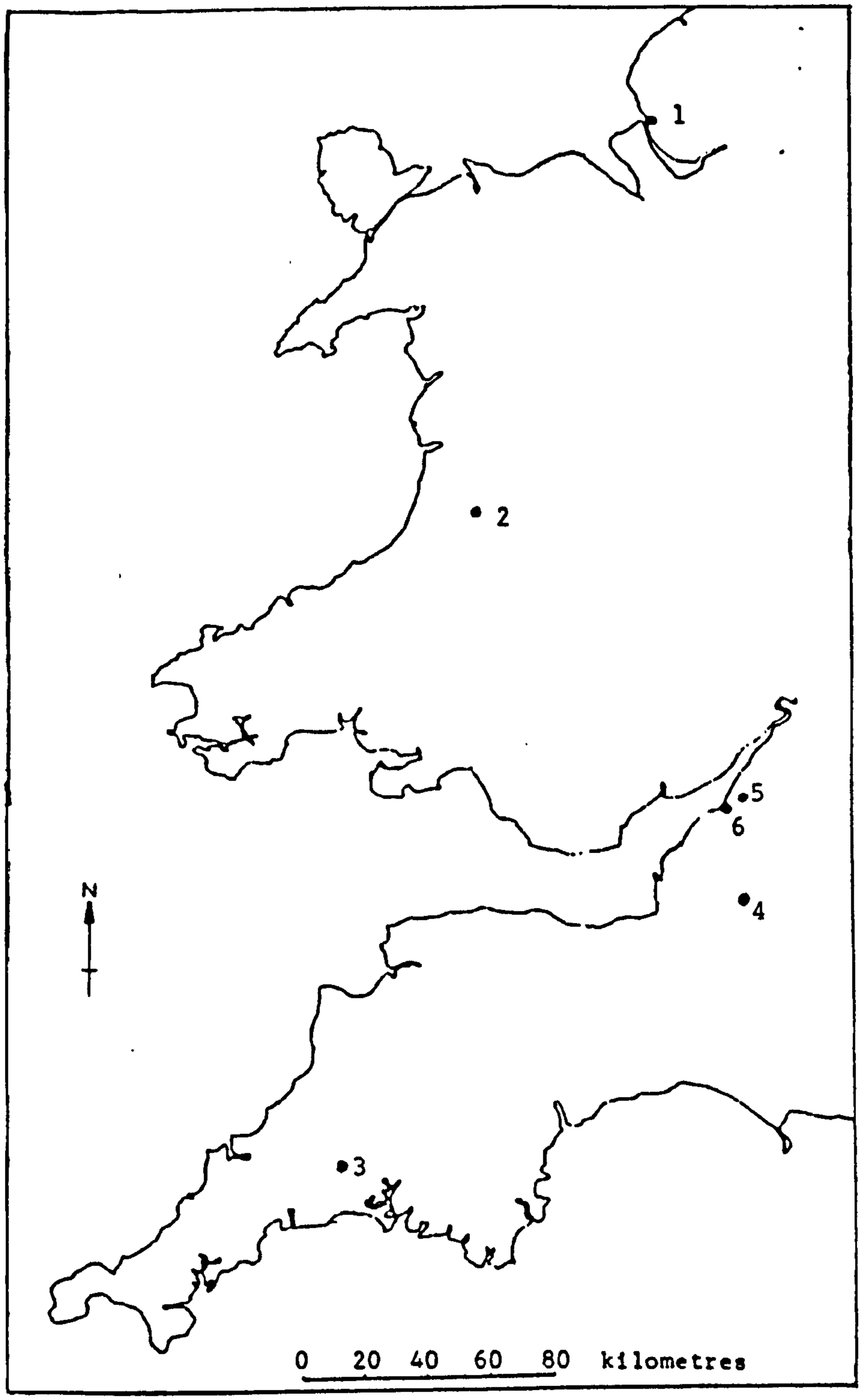
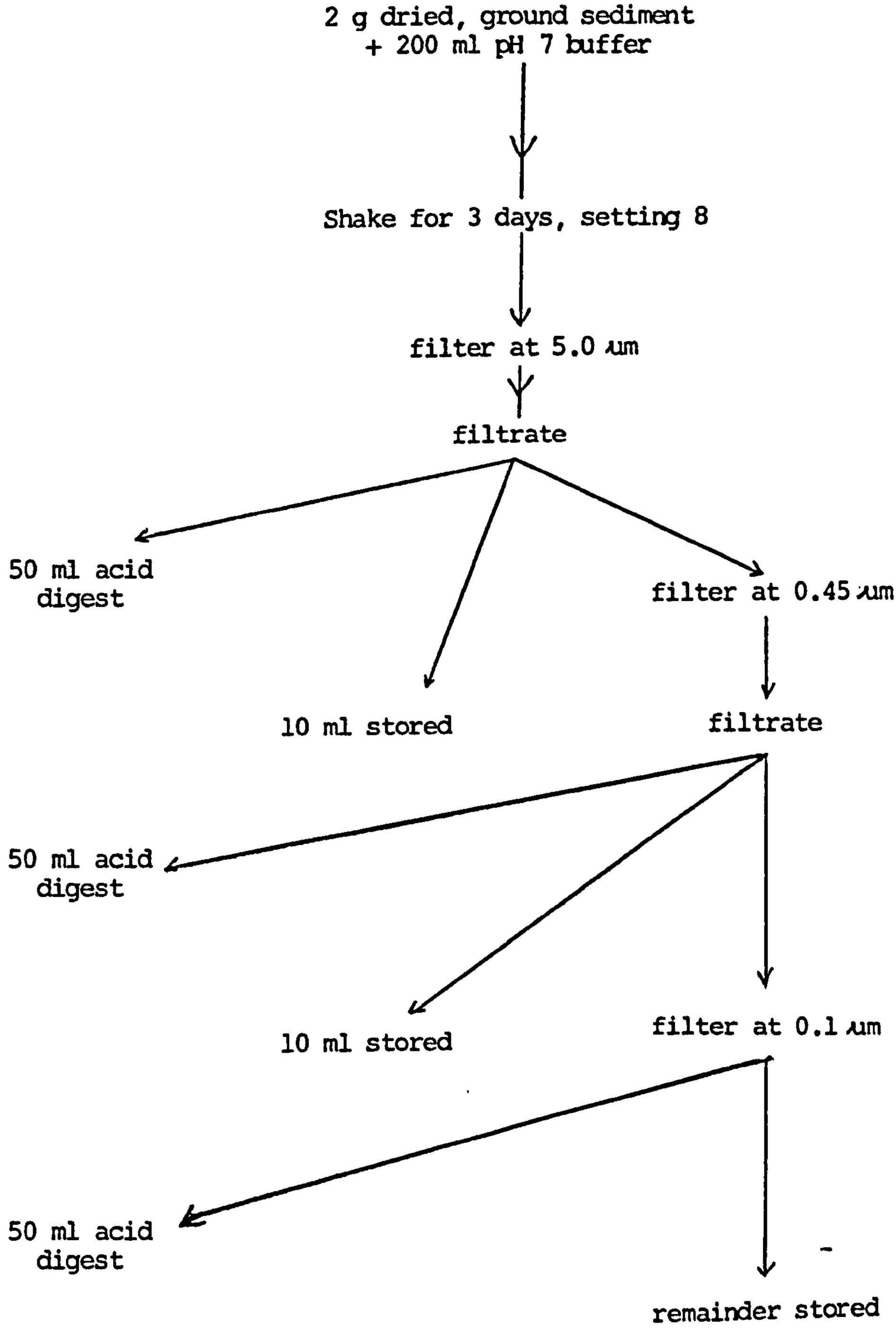


Figure 31 : Location of sediment sampling sites

KEY :
1. Liverpool
2. A. Ystwyth
3. R. Caradon
4. Mineries Pool
5. Lawrence Weston
6. Avonmouth

Figure 32 : Fractionation of metals in sediments by change of pH and by filtration



Carried out at pH 5 and pH 3, also.

Table 40 : Mineries Pool Sediment fractionated by pH and filtration.

Analysis by DPASV

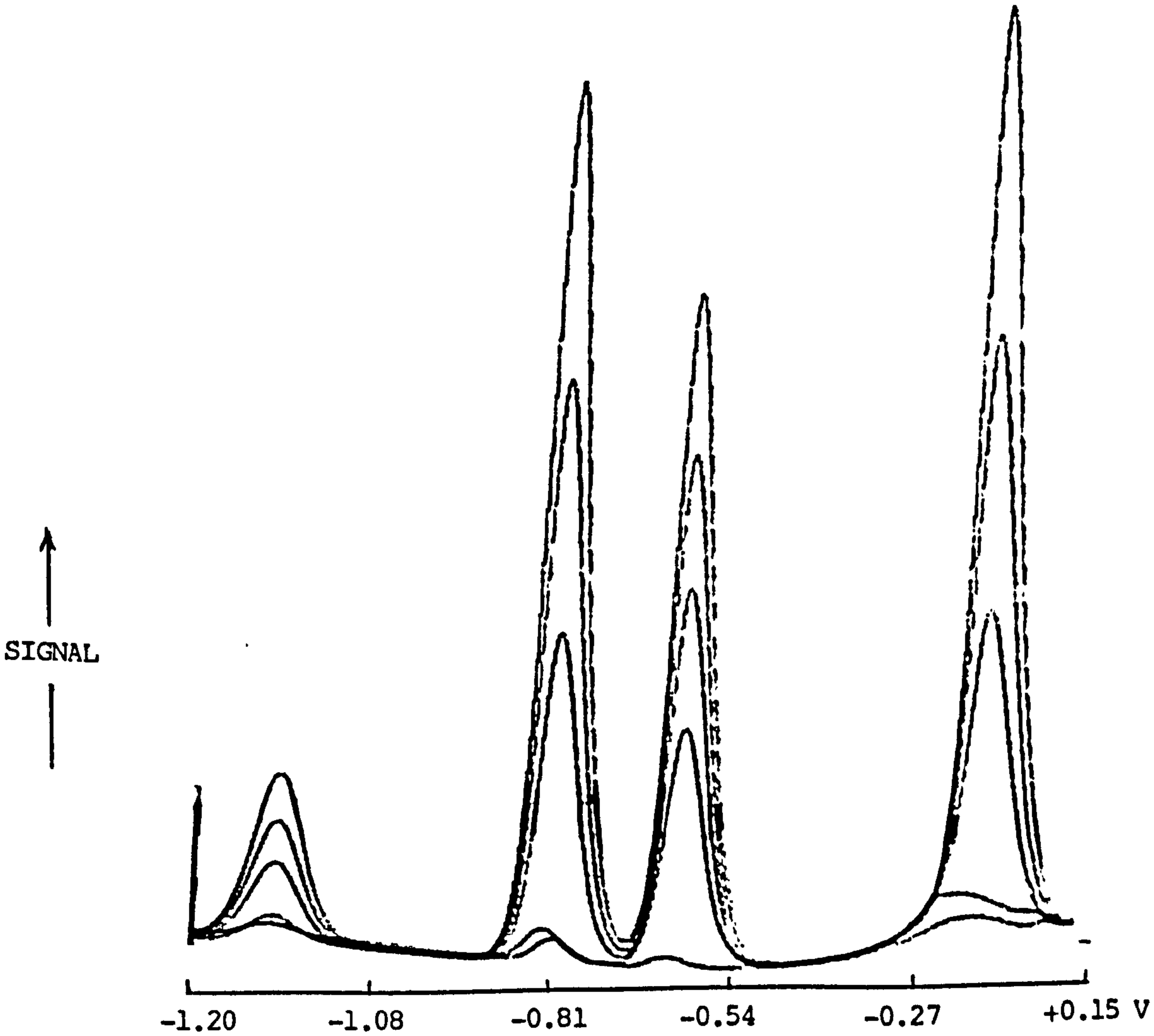
	Pb concentration µg/g (DW)			
Size fraction (µm)	pH 7	pH 5*	pH 3*	pH 3 (acid digest)*
< 5.0	177	25.3	34.5	39.7
< 0.45	393	29.0	93.8	96.4
< 0.1	2398	27.0	31.1	31.8

* Values x 10^3

Table 41 : Caradon sediment, fractionated by pH and filtration.

	Cu concentration µg/g (DW)			
Size fraction (µm)	pH 7	pH 5	pH 3	pH 3 (acid digest)
< 5.0	167	505	1621	1668
< 0.45	52	334	633	646
< 0.1	41	313	591	546

Figure 33 : Differential pulse stripping curve (HMDE) of Zn, Cd, Pb and Cu in 4% v/v Mineries Pool sediment extract < 0.45 µm : 0.2 M ammonium citrate



Mersey samples : Garston Docks sediment fractionation

The samples were divided into three fractions by wet sieving through 40 µm and 20 µm mesh polyester sieves (Simonester, Polyester precision textiles 31260 (P.E. 40 & P.E. 20) Swiss made). The fractions were dried at 110°C, weighed and acid digested with 10 ml concentrated nitric acid. Evaporation to 2 ml was carried out by heating under an IR lamp. A blank was treated similarly. After cooling, the samples were filtered through Whatman 541 filter paper and made up to a final volume of 50 ml with DDW. Determinations for Cd, Cu, Pb and Zn were carried out using an ILL 151 atomic absorption spectrometer, with the conditions given on page 151. The results obtained are presented in Tables 42 to 45.

Table 42 : Samples from Garston Docks : Zinc

Sample number	Mesh size (µm)	Zinc µg/g (dry weight)
1	> 40	228
	20-40	153
	< 20	250
2	> 40	228
	20-40	269
	< 20	301
3	> 40	301
	20-40	274
	< 20	256
4	> 40	266
	20-40	283
	< 20	291

Table 43 : Samples from Garston Docks : Cadmium

Sample Number	Mesh size (um)	Cadmium ug/g (dry weight)
1	> 40	1
	20-40	1
	< 20	4
2	> 40	1
	20-40	4
	< 20	5
3	> 40	4
	20-40	3
	< 20	3
4	> 40	3
	20-40	3
	< 20	1

Table 44 : Samples from Garston Docks : Copper

Sample number	Mesh size (um)	Copper ug/g (dry weight)
	> 40	47
1	20-40	33
	< 20	151
	> 40	49
2	20-40	399*
	< 20	1451*
	> 40	113
3	20-40	130
	< 20	115
	> 40	143
4	20-40	156
	< 20	135

* These results are an average of several samples - sediment appears grossly contaminated in the finer parts.

Table 45 : Samples from Garston Docks : Lead

Sample number	Mesh size (μm)	Lead μg/g (dry weight)
	> 40	88
1	20-40	62
	< 20	295
	> 40	77
2	20-40	220
	< 20	212
	> 40	183
3	20-40	240
	< 20	191
	> 40	227
4	20-40	267
	< 20	211

Ystwyth Rock sample

The rock sample was finely ground by means of a Tema mill (Tema (Machinery) Ltd., Banbury, Laboratory disc mill; 1000 rpm, 50 cycles). The ground rock (2 g) was mechanically shaken with 200 ml of pH 7 (0.2 M ammonium acetate) background electrolyte, for 3 days. The solution was filtered through a 5.0 μm membrane filter and analysed by DPASV, using the conditions outlined on page 150 . The results are given in Table 46. An acid digest was also carried out on this solution.

Table 46 : Ystwyth rock sample at pH 7 and acid digestion of the pH 7 extraction

Sample	Size fraction (µm)	Concentration µg/ml			
		Zn	Cd	Pb	Cu
Y_4 pH 7	< 5	108.0	N.D.	38.4	N.D.
Y_4 Acid digest	< 5	208.7	N.D.	42.7	N.D.

Slag samples

The slag samples were finely ground by means of a Tema mill and were then sieved through a 500 µm mesh sieve. Acid digests were carried out on 0.5 g of each of the samples. Analysis was carried out by DPASV, using a 90 s deposition time, 0.1 mA current and standard additions (20 µl) of 0.2 µg/ml. The results are given in Table 47.

Table 47 : Analysis of slag samples (acid digests) by DPASV

Sample	Concentration µg/g			
	Zn	Cd	Pb	Cu
Slag 66 BT	20.9	N.D.	8.4	183.6
Slag 65 BT	43.9	N.D.	4.0	49.8
Slag 64 BT	27.8	N.D.	N.D.	35.8

e) Apparatus and Instrumental Parameters

See Part II, Section I.

SECTION 3 : SOILS : METAL FRACTIONATION STUDIES

3.1 Experimental Preparation

a) Reagents

b) Site description

c) Sample collection

d) Sample treatment, Analysis and Results

e) Apparatus and Instrumental Parameters

SECTION 3 : SOILS: METAL FRACTIONATION STUDIES

3.1 Experimental Preparation

a) Reagents

AristaR nitric, acetic and hydrofluoric acid. AnalaR ammonium acetate, cadmium, copper, lead and zinc nitrates, magnesium chloride, sodium acetate, hydroxylamine, 30% (w/v) hydrogen peroxide, 0.91 ammonia solution, citric acid, sodium hydroxide, ammonium chloride, ascorbic acid.

Preparation of stock solutions

Background electrolyte pH 2.5 : A mixture of AnalaR 1 M ammonium chloride, 0.1 M citric acid and 0.025 M ascorbic acid was prepared by dissolving 53.5 g of ammonium chloride and 21 g of citric acid in 600 ml of DDW. Ascorbic acid (4.5 g) was added to the solution, before being diluted to a final volume of 1000 ml.

5×10^{-2}M mercuric nitrate solution : Triply distilled mercury (1 g) was dissolved in 6.5 ml of 10% v/v Aristar nitric acid. The solution was evaporated to fumes by heating under an infra red lamp. The residue was dissolved in DDW and made up to a final volume of 100 ml.

b) Site description

Soil samples were collected from Hallen Wood (ST 555 802), Shipham (ST 441 571), Charterhouse (St 506 561), Royal Fort Gardens, Bristol University (ST 581 734) and the Computer Centre, Bristol University (ST 581 733), in Avon and Somerset. Soil was also collected at Luckett (SX 385 736) and Callington (SX 368 693) in Cornwall. A soil sample from Bedminster (ST 576 713) was supplied by Dr. D. Roberts, School of Chemistry, University of Bristol.

Samples of organic woodland litter were collected from Hallen (ST 555 802) and Wetmoor (ST 746 878) woods.

Wetmoor is situated 24 km northeast of Avonmouth and receives relatively little "fall out" from the Avonmouth lead-zinc smelter. The deciduous woodland at both Wetmoor and Hallen is on heavy clay soil derived chiefly from blue-grey calcareous clay.

The Royal Fort Gardens and Computer Centre samples from Bristol University, in the centre of Bristol, are not immediately adjacent to any major roads. The Royal Fort Garden site is separated from a car parking area by high walls, whereas the Computer Centre site was not. These two sites were chosen to provide a "background" level for the City.

The Bedminster sample was obtained from a garden intended for vegetable produce. However, many places in and around Bristol were associated with the smelting of copper and brass manufacture (282). The Bristol Brass Wire Company began in 1702. One of the iron and brass works was on the outskirts of Bedminster (ST 597 727) producing copper, spelter, wire and patent zinc and iron (283,284). The industry closed in 1927, but the remains of smelting can be seen in the form of black building blocks made of copper slag (284). Lead shot has also been manufactured in the area, beginning in 1785 and lasting until 1968 (285).

Calamine was first discovered in Somerset in 1566 and a century later it was being mined in many places on the West Mendips, including Shipham. Contamination resulting from the mining activities is exemplified by reclaimed surface soils in Shipham. Mining activities ended at the beginning of the nineteenth century.

Charterhouse, on the Mendip Hills, was mined for lead by the Romans. Mining continued here until the early 1900s. The Roman slag was resmelted at the Charterhouse works between 1824 and 1908. The slag and mining waste remains.

The Callington and Tavistock district includes the western and southern margins of the Dartmoor granite and extends westward to include the two small granite masses of Gunnislake and Kit Hill. Mines in this area produced large yields of copper and pyrite. Tin, mispickel, silver, lead and wolfram have also been found here. Some of the dumps were worked over during the period 1914 to 1920 and parts of the Redmoor mine were in use in 1943, but mining had largely been abandoned by this time. The dumps, however, remain.

The location of the sampling sites is indicated in Figure 34.

c) Sample collection

The samples were transferred to the laboratory in polyethylene bags.

d) Sample treatment, analysis and results

Model samples : contaminated peat

A range of contaminated peat samples were prepared. A 5 cm (i.d.) glass column was plugged with glass wool and filled with Levington's Universal Mixture to a height of 42 cm. A 0.1 M solution of the required metal was prepared by dissolving the salt in pH 7, 0.2 M ammonium acetate. The resultant solution was passed through the column over a period of 7 days. The column was allowed to drain, before being washed with several applications of DDW. The peat was removed from the column and dried at 50°C. The dried peat was ground and sieved through a 500 µm mesh.

A 'four metal' peat containing cadmium, copper, lead and zinc was prepared in this way. A 'copper-peat' and 'lead-copper' peat were also prepared.

The acid extractable content for the 3 peat samples was determined by digesting 1 g of each sample with 5 ml concentrated HNO_3 and 5 ml concentrated HF in a polypropylene centrifuge tube. The mixture was evaporated

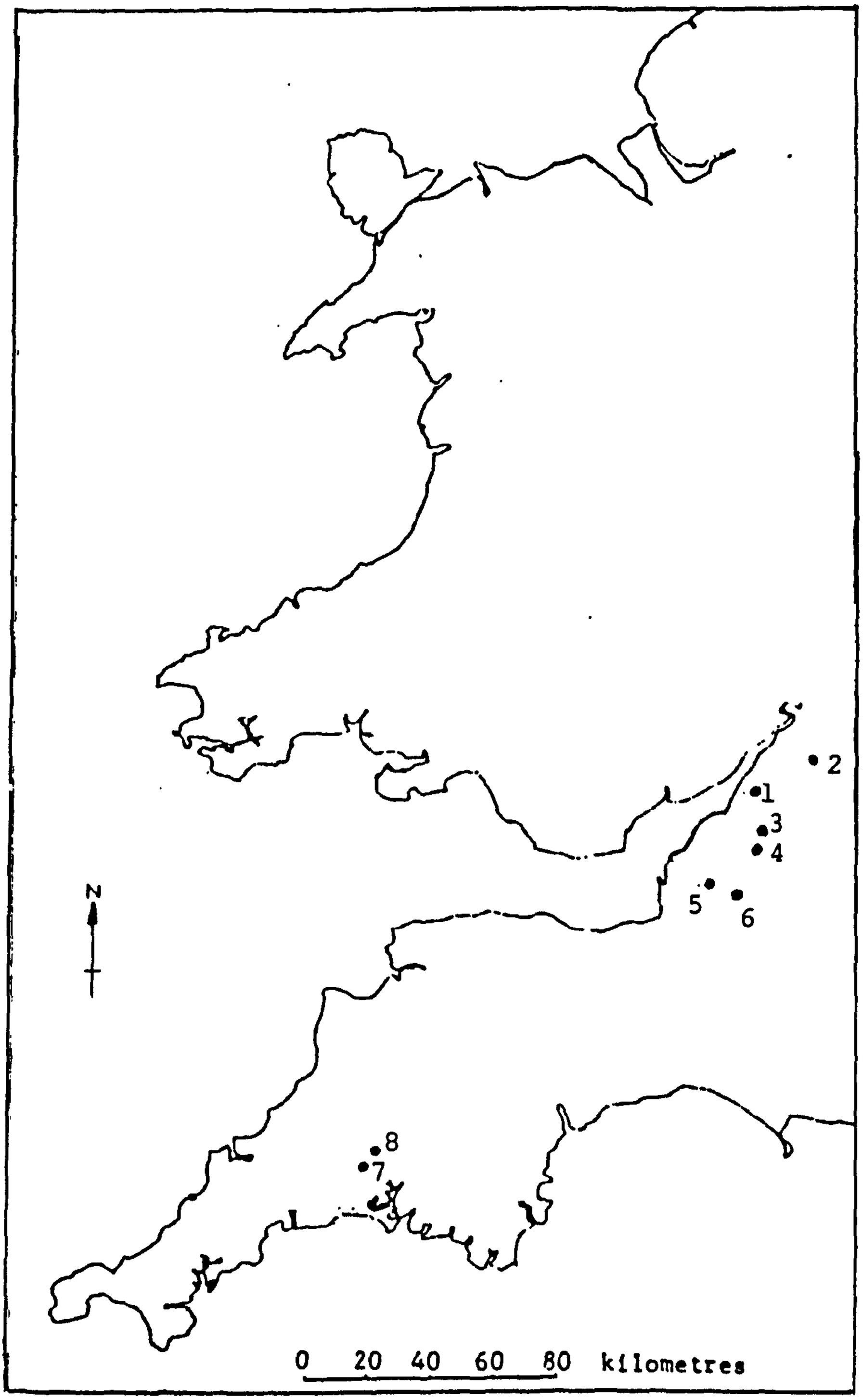


Figure 34 : Location of sampling sites

KEY :
1. Hallen Wood
2. Wetmoor Wood
3. University of Bristol
4. Bedminster
5. Shipham
6. Charterhouse
7. Callington
8. Luckett

to approximately 2 ml by heating in a water bath. The Cu-peat was also digested with nitric acid as described in Section 2 (Part II). On cooling the mixture was filtered through a Whatman 541 filter paper. The filtrate was diluted to a suitable volume with DDW. Analysis was carried out by use of an ILL 151 atomic absorption spectrometer. The results are given in Tables 48, 49 and 50.

The Tessier et al. (218) extraction scheme, with some modification, was performed on each of the peat samples. The scheme most often used is outlined in Figure 35. The sequential extraction procedure was applied to 1 g of each sample using 50 ml polypropylene centrifuge tubes, with a mechanical shaker to mix the solutions as necessary. The mixtures were centrifuged at 3000 rpm for 30 minutes, and where necessary were also filtered through Whatman 541 filter papers. The residual solids obtained from each extraction were washed with 8 ml DDW.

In the Tessier et al. scheme, examination of the reducible fraction is carried out before the organic fraction. The sequence was applied to the Pb/Cu peat sample. A soluble fraction was not determined. The organic then reducible sequence was determined on one of the two samples. The organic-reducible sequence and a soluble fraction were carried out on the 'four metal' peat sample.

The extracts were examined by FAAS, or DPASV using the conditions outlined on page 151. The results obtained are given in Tables 48, 49 and 50 and a voltammogram of the exchangeable fraction of the Cu-peat sample is given in Figure 36.

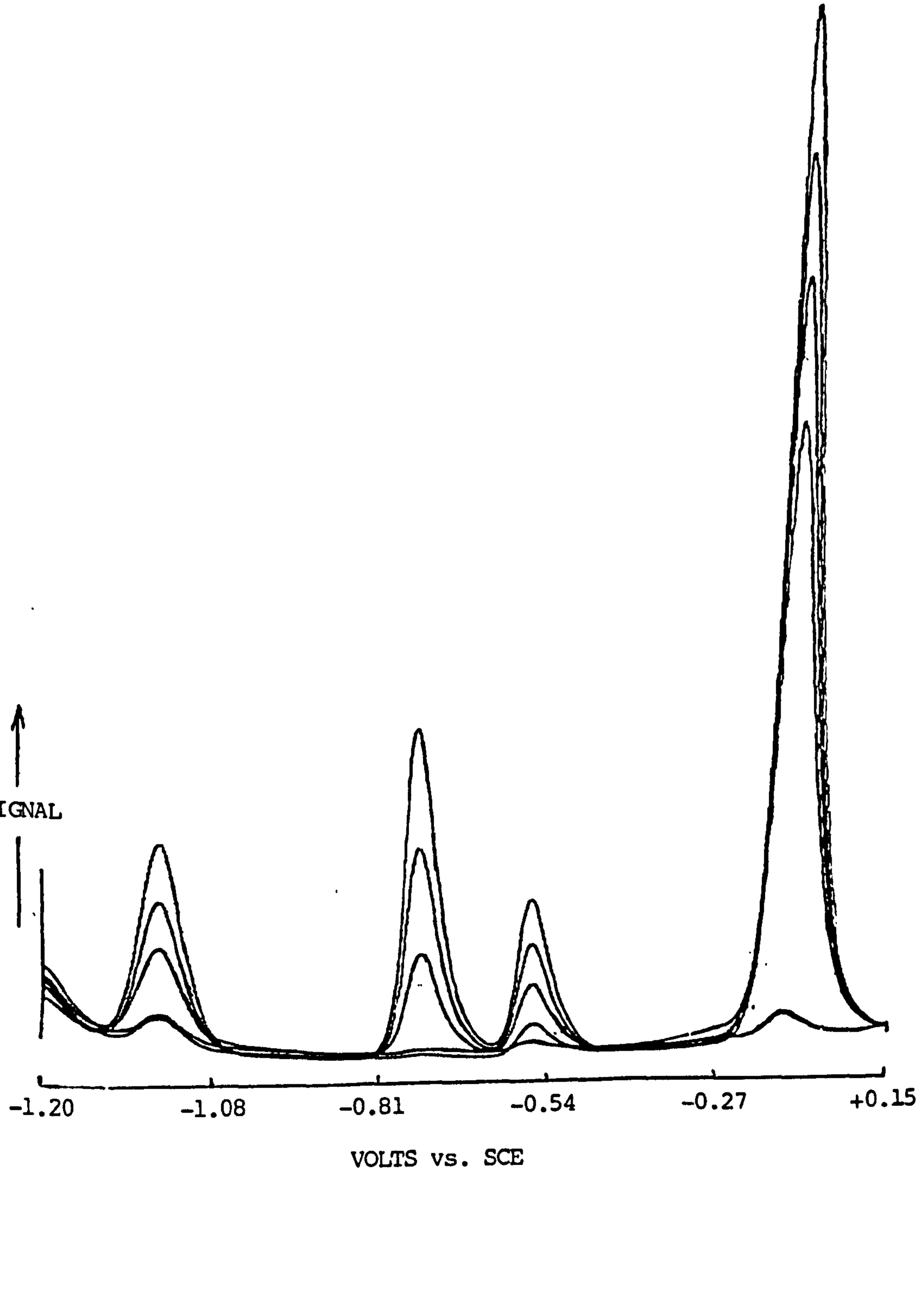
The fractions were obtained as follows :-

Water soluble : The sample (1 g) was extracted with 8 ml of DDW for 1 hour at room temperature, with continuous agitation. After centrifugation the extract was made up to 25 ml with DDW and stored in a polythene container.

Figure 35 : Sequential extraction procedure for the fractionation of heavy metals (After Tessier, Campbell and Bisson (241))

PROCEDURE	FRACTION
1 g dried sample	
↓	
8 ml DDW	
Shaken 1 hour. R.T.	——— Water soluble
↓	
8 ml 1 M $MgCl_2$	
Shaken 1 h. R.T.	——— Exchangeable
↓	
8 ml 1 M NaOAc	
Shaken 5 h. R.T.	——— Carbonate
↓	
3 ml 0.002 M HNO_3	
5 ml 30% H_2O_2 at 85°C for 2 h.	
3 ml H_2O_2 at 85°C for 2 h.	——— Organic
5 ml 3.2 M NH_4OAc in 20% HNO_3.	
Diluted to 20 ml with DDW	
and shaken 30 min	
↓	
20 ml 0.04 M $NH_2OH.HCl$ in 25%	
acetic acid at 96°C	——— Reducible
↓	
5 ml HF, 3 ml HNO_3, 3 ml HNO_3	
sequential digestion	——— Residual

Figure 36 : Differential pulse stripping curve (HMDE) for Zn, Cd, Pb and Cu in 0.1% v/v Cu contaminated peat (Exchangeable Fraction) : 0.2 M ammonium citrate



Exchangeable : The residue from the soluble fraction was leached with 8 ml of 1M $MgCl_2$ (pH 7) for 1 hour at room temperature with continuous agitation. After centrifugation, the decanted supernatant was diluted to 25 ml with DDW.

Carbonate : The residue from the exchangeable fraction was leached with 8 ml of 1 M NaOAc adjusted to pH 5.0 with HOAc, at room temperature and with continuous agitation for 5 hours. After centrifugation the supernatant was decanted and diluted to 25 ml with DDW.

Reducible : The residue from the carbonate fraction was extracted with 20 ml of 0.04 M $NH_2OH.HCl$ in 25% (v/v) HOAc. The extraction was performed at 96 $\pm$ 3°C in a water bath for 6 hours, with occasional agitation. After centrifugation the decanted supernatant was diluted to 25 ml with DDW.

Organic : The residue from the reducible fraction was extracted with 3 ml of 0.02 M HNO_3 and 5 ml of 30% (w/v) H_2O_2. The mixture was heated to 85 $\pm$ 2°C in a water bath for 2 hours with occasional agitation. A second 3 ml aliquot of 30% H_2O_2 was then added and the sample again heated to 85 $\pm$ 2°C in a water bath for 2 hours with occasional agitation. After cooling, 5 ml of 3.2 M NH_4OAc in 20% (v/v) HNO_3 (pH 3.4) was added and the sample diluted to 20 ml with DDW. The mixture was continuously agitated for 30 minutes. The extract obtained was diluted to 25 ml with DDW.

Residual : The residue from the organic fraction was sequentially digested with 5 ml HF, 3 ml HNO_3 and a second aliquot of 3 ml HNO_3. After centrifugation the extract was diluted to 25 ml with DDW.

Lead contaminated ferric hydroxide

Ferric hydroxide precipitated by addition of aqueous ammonia :-

$$Fe^{3+} + 3NH_3 + 3H_2O \longrightarrow Fe(OH)_3(S) + 3NH_4^+$$

is an example of a gelatinous precipitate. However, in principle a very pure, dense precipitate can be produced from homogeneous solution, by the addition of urea and succinic acid and then boiling the solution. In practice, this process was found to be time consuming, producing a small quantity of the precipitate which was difficult to filter. Greater success was achieved by isothermal distillation :-

A clean polypropylene beaker containing 100 ml of 25% v/v ammonia solution was placed in a large glass vacuum desiccator. A second beaker containing a mixture of 250 ml solution of ferric and lead nitrates was also placed in the desiccator. The closed system was left overnight at room temperature. The precipitate obtained was filtered using glass sinter Number 4.

The lead contaminated ferric hydroxide thus produced was subjected to the fractionation scheme outlined on page 173 (i.e. the reducible fraction before the organic extraction). A further sample was subjected to the scheme outlined in Figure 35 (i.e. the organic fraction before the reducible fraction). Analysis of the fractions was carried out by FAAS (using the conditions given on page 151) and by DPASV, using a current of 0.1 mA, 60 s deposition and 1 µg/ml Pb spikes. The results obtained are given in Table 51 and a voltammogram of the Pb/$Fe(OH)_3$ exchangeable fraction is given in Figure 37.

The scheme (Figure 35) was also applied to the National Bureau of Standards, Standard Reference Material 718 Orchard leaves (Table 52).

Table 48 : Fractionation of Pb/Cu peat. Analysis by FAAS

	Concentration (µg/ml)			
Fraction	Zn	Cd	Pb	Cu
Exchangeable	N.D.	1	233	1790
Carbonate	N.D.	1	383	3278
Reducible	N.D.	N.D.	833	6973
Organic	120	N.D.	135	187
Residual	N.D.	N.D.	458	1020
Total	120	2	2042	13248
Acid digest (HF/HNO_3)	120	2	2066	13228

N.B. (Reducible fraction before organic fraction)

Table 49 : Fractionation of Cu peat. Analysis by DPASV (I) & AAS (II)

	Cu Concentration (µg/ml)	
Fraction	I	II
Soluble	*	132
Exchangeable	1186	806
Carbonate	3113	3384
Organic	4967	4529
Reducible	1853	2513
Residual	306	408
Total	11425	11772
Acid digest (HF/HNO_3)	11880	11908
Acid digest (HNO_3)	10760	10758

* No soluble fraction carried out.

Table 50 : Fractionation of "4 Metal" peat. Analysis by FAAS

	Concentration (ug/ml)			
Fraction	Zn	Cd	Pb	Cu
Soluble	N.D.	24	32	N.D.
Exchangeable	N.D.	22	5159	N.D.
Carbonate	N.D.	18	613	N.D.
Organic	811	43	8456	6091
Reducible	25	16	4343	1820
Residual	35	6	3590	688
Total	871	129	22193	8599
Acid digest (HF/HNO_3)	898	228	22981	8582

Table 51 : Fractionation of lead contaminated ferric hydroxide. Analysis by FAAS and DPASV

	Pb Concentration ug/ml			
Fraction	DPASV I	AAS I	DPASV II	AAS II
Soluble	9909	7281	9997	6623
Exchangeable	114	151	120	120
Carbonate	150282	150284	150567	150559
Organic	91161	125050	19994	21877
Reducible	28488	51402	209913	200998
Residual	64417	64435	4999	5686
Total	344371	398603	395590	385863
Acid digest (HF/HNO_3)	313802	407990	395454	406208

I = Organic fraction before Reducible

II = Reducible fraction before Organic

Figure 37 : Differential pulse stripping curve (HMDE) for Pb in Pb contaminated ferric hydroxide (Exchangeable Fraction): 0.2 M ammonium citrate

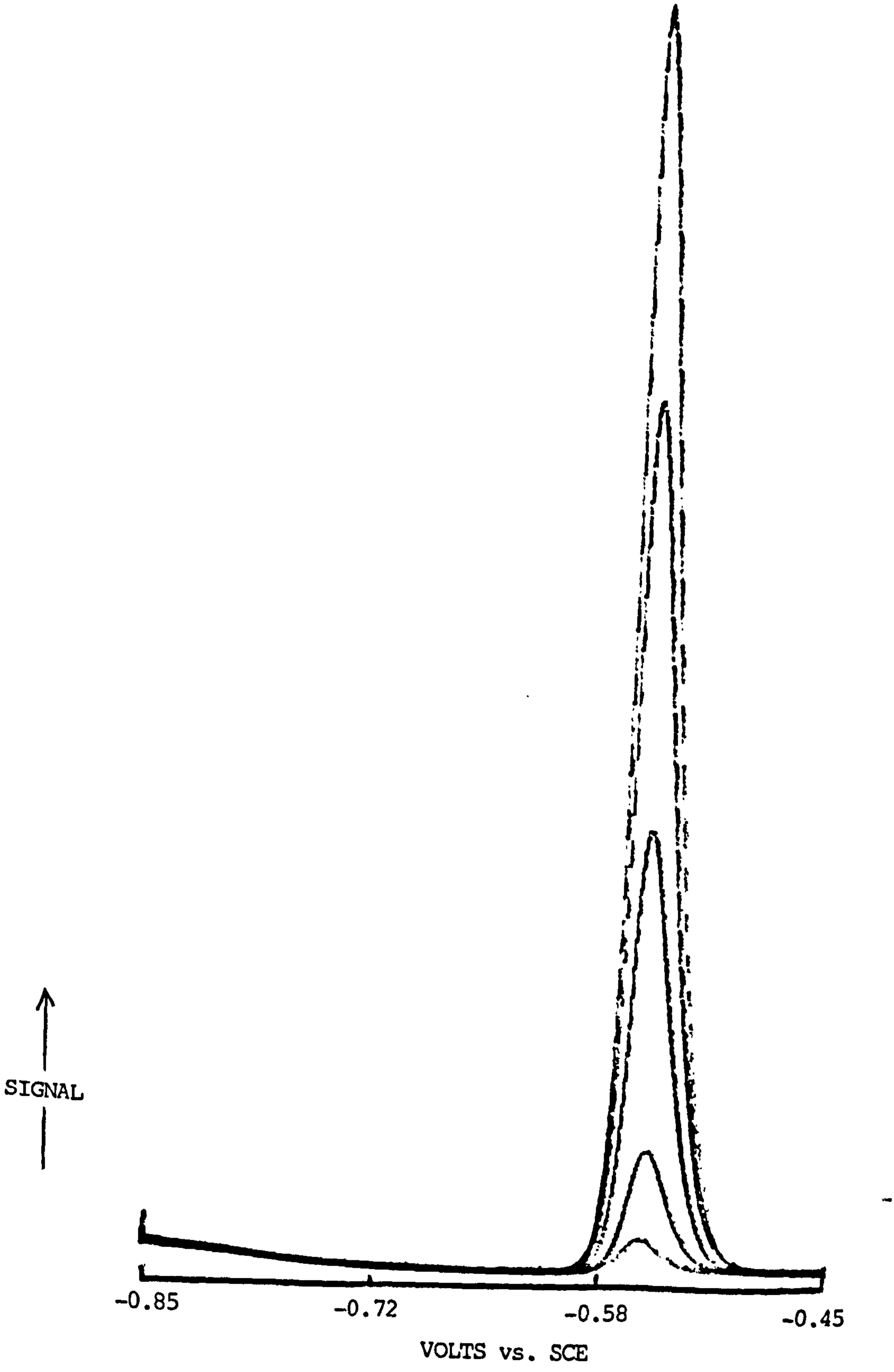


Table 52 : Fractionation of SRM 718 Orchard leaves. Analysis by FAAS (Acid digest (HF/HNO_3) by AAS and DPASV-HMDE)

	Concentration µg/g (DW)			
Sample	Zn	Cd	Pb	Cu
Soluble	2.6	N.D.	N.D.	N.D.
Exchangeable	6.9	0.5	N.D.	N.D.
Carbonate	2.6	N.D.	4.4	2.4
Organic	11.1	N.D.	3.4	5.7
Reducible	N.D.	N.D.	10.5	N.D.
Residual	4.7	N.D.	5.1	N.D.
Total	27.9	0.5	23.4	8.1
AAS Acid digest	17.8	0.1	42.3	10.4
HMDE Acid digest	*	N.D.	28.1	9.4
NBS Value	25.0	0.11	45	12

* Interference

Organic litter and soil samples

The litter and soil samples were dried at 50°C, before being ground to pass a 500 µm sieve. Acid digests were carried out and examined by FAAS. The litter samples were fractionated according to the scheme given in Figure 35. The determinations were carried out by DPASV as described previously (page 150). The zinc determination was by FAAS. The results obtained are given in Table 53.

The Luckett, Callington and Hallen soil samples were subjected to the scheme on page 171 and the fractions were examined by FAAS.

A Bedminster acid digest was also studied by DPASV at a HMDE (0.2 M ammonium citrate pH 3) and by DPASV at an RDE at pH 2.5 (1 M ammonium

chloride, 0.1 M citric acid, 0.025 M ascorbic acid) and at pH 3 (0.2 M ammonium citrate) (Table 57 and Figures 38 and 39).

Table 53 : Fractionation of Hallen and Wetmoor Woodland litter. Analysis by DPASV. (Zinc analysis by FAAS)

a) Hallen

Fraction	Concentration (ug/g) Zn	Cd	Pb	Cu
Soluble	N.D.	N.D.	N.D.	N.D.
Exchangeable	326	36	423	N.D.
Carbonate	65	5	163	6
Organic	180	70	1568	198
Reducible	944	N.D.	3807	59
Residual	2728	34	1499	20
Total	4243	145	7460	283
Acid digest (HF/HNO_3)	4380	142	7430	272

b) Wetmoor

Fraction	Concentration (ug/g) Zn	Cd	Pb	Cu
Soluble	1	N.D.	4	N.D.
Exchangeable	16	3	33	N.D.
Carbonate	2	N.D.	10	N.D.
Organic	8	23	22	7
Reducible	82	N.D.	18	5
Residual	61	13	35	21
Total	170	39	122	33
Acid digest (HF/HNO_3)	233	30	119	33

Table 54 : Fractionation of Luckett and Callington soil samples.

Analysis by FAAS. a) Zn and Cd

	Zn Concentration µg/g (DW)					
Fraction	C1	C2	C3	L1	L2	L3
Soluble	2	1	N.D.	6	N.D.	N.D.
Exchangeable	67	98	76	7	1	N.D.
Carbonate	5	12	6	N.D.	N.D.	N.D.
Organic	88	86	55	N.D.	2	N.D.
Reducible	64	71	37	N.D.	2	4
Residual	194	226	334	309	300	342
Total	420	494	508	322	305	346
Acid digest (HF/HNO_3)	528	539	579	488	372	430

Cd Concentration µg/g (DW)

Sample	Sol.	Exch.	Carb.	Org.	Red.	Res.	Tot.	A.D.
C2	N.D.	1	1	N.D.	N.D.	N.D.	2	5

Cd Concentration µg/g (DW)

Fraction	C1	C3	L1	L2	L3
Acid digest (HF/HNO_3)	7	1	5	3	5

Cd was not detected in any fraction of C1, C3, L1, L2 or L3.

C1 = Callington soil at a depth of ~ 8 cm.

C2 = Callington soil beneath Agrostis tenuis at a depth of ~ 4 cm.

C3 = Callington soil beneath Rhy tidiadelphus squarrosus (~1 cm).

L1 = Luckett soil at the surface (0.0 cm depth)

L2 = Luckett soil beneath Agrostis canina (~4 cm depth).

L3 = Luckett soil beneath Ceratodon purpureus (~1 cm).

Table 54 continued. b) Pb and Cu

	Pb Concentration ug/g (DW)					
Fraction	C1	C2	C3	L1	L2	L3
Soluble	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Exchangeable	N.D.	3	11	N.D.	N.D.	N.D.
Carbonate	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Organic	89	54	139	N.D.	N.D.	N.D.
Reducible	292	8	97	N.D.	N.D.	N.D.
Residual	821	193	72	164	126	193
Total	1202	258	319	164	126	193
Acid digest (HF/HNO_3)	1498	843	489	245	238	216

	Cu Concentration ug/g (DW)					
Fraction	C1	C2	C3	L1	L2	L3
Soluble	4	1	N.D.	6	N.D.	N.D.
Exchangeable	11	17	1	10	N.D.	1
Carbonate	1	4	N.D.	N.D.	N.D.	N.D.
Organic	333	339	112	35	14	8
Reducible	128	70	41	4	32	38
Residual	387	533	237	497	232	253
Total	864	964	391	552	278	300
Acid digest (HF/HNO_3)	1178	982	440	581	291	333

Table 55 : Fractionation of Hallen Soil Sample. Analysis by FAAS

	Concentration µg/g (DW)			
Fraction	Zn	Cd	Pb	Cu
Soluble	N.D.	N.D.	N.D.	N.D.
Exchangeable	59	14	N.D.	N.D.
Carbonate	4	N.D.	N.D.	N.D.
Organic	609	10	101	21
Reducible	610	1	115	4
Residual	353	N.D.	27	12
Total	1635	25	243	37
Acid digest (HF/HNO_3)	1677	26	260	40

Table 56 : Somerset and Avon soil samples. Acid digests analysed by FAAS

Sample	Concentration µg/g (DW) Zn	Cd	Pb	Cu
RFG1	140	N.D.	120	49
RFG2	132	N.D.	120	53
Mean	136	-	120	51
CC1	794	1	270	105
CC2	796	1	268	105
Mean	795	1	269	105
SD1	73100	524	5048	70
SD2	72900	334	5072	82
Mean	73000	429	5060	76
S1	21322	100	3265	44
S2	20726	54	3269	38
Mean	21024	77	3267	41
SG1	-	4	385	-
SG2	-	6	395	-
Mean	-	5	390	-
Ch1	3321	61	2995	36
Ch2	3323	57	3041	22
Mean	3322	59	3018	29
B1	1122	39	2443	221
B2	-	47	2359	-
Mean	-	43	2401	-

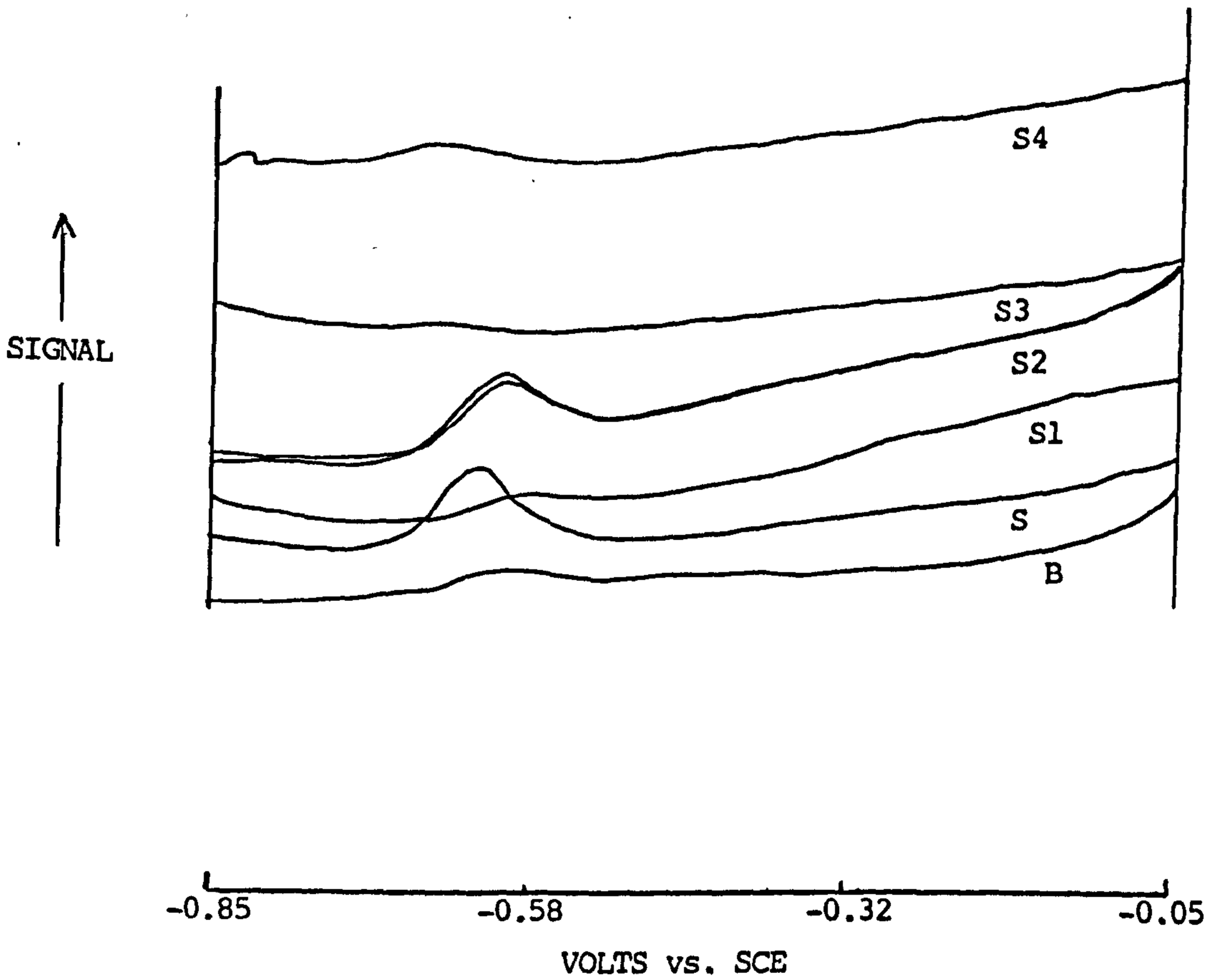
RFG = Royal Fort Gardens
SD = Shipham soil at a depth ~ 10 cm
SG = Shipham Garden soil
B = Bedminster
CC = Computer Centre
S = Shipham soil at a depth ~ 0 cm
Ch = Charterhouse

Table 57 : Bedminster soil sample acid digest. Analysis by FAAS and DPASV (at HMDE and RDE)

Sample	Concentration µg/g (DW) Zn	Cd	Pb	Cu
B1 AAS	1123	39	2443	221
B2 AAS	-	47	2358	-
B1 HMDE	1552	78	2173	78
B1 RDE	I	I	I	I

I = interference

Figure 38 : Differential pulse stripping curve (RDE) of Cd, Pb and Cu in 1% v/v Bedminster soil (Acid Digest) : 0.2 M ammonium citrate

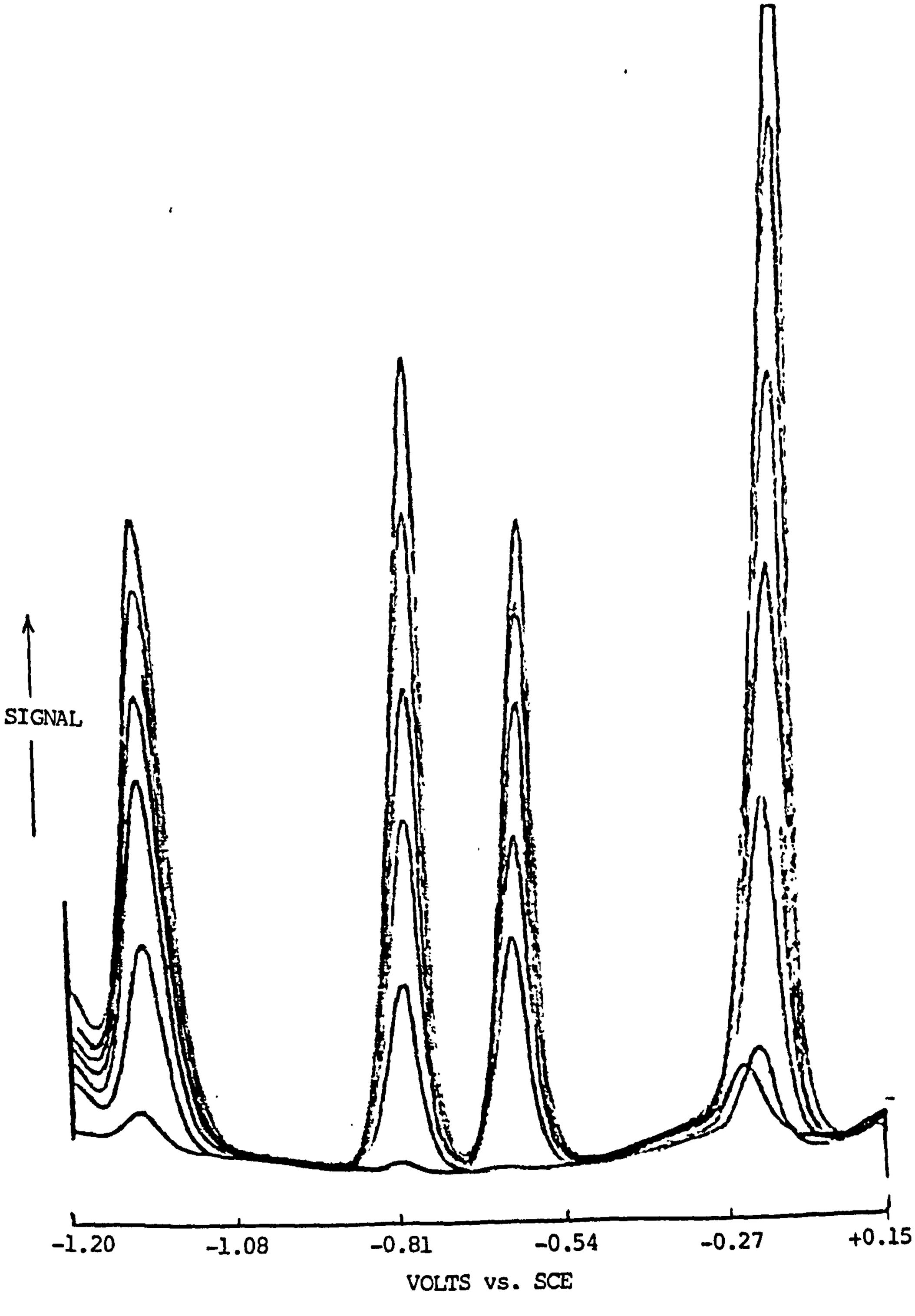


B = Buffer

S = Sample

S1, S2, S3 & S4 = standard additions

Figure 39 : Differential pulse stripping curve (HMDE) for Zn, Cd, Pb and Cu in 0.2 M ammonium citrate (acid digest) : Bedminster soil



"Quick" fractionation scheme

The "4 metal" peat prepared earlier was fractionated using the scheme given in Figure 40. The sequential extraction procedure was applied to 0.5g of the sample, in a 50 ml polypropylene centrifuge tube. FAAS (conditions, page 151) was used for the examination of the fractions. The results are given in Table 58.

The fractions were obtained as follows :-

Exchangeable/Carbonate : 0.5 g of the sample was extracted with 8 ml of 0.2 M citric acid (pH 2.5) for 1 hour at room temperature with continuous agitation. After centrifugation the extract was diluted to 25 ml with DDW.

Reducible : The reducible extraction was carried out as described on page 173.

Organic : The residue from the reducible fraction was extracted with 20 ml of 0.44 M NaOH for 1 hour, with occasional agitation. After centrifugation the extract was made up to 25 ml with DDW.

Residual : The residue from the organic fraction was sequentially digested as described on page 173.

The residual solids obtained from each extraction were washed with 8 ml of DDW.

The scheme was also applied to Standard Reference Material 718 Orchard leaves (Table 59).

Figure 40 : "Quick" Fractionation Scheme

PROCEDURE		FRACTION
0.5 g dried sample		
↓		
8 ml 0.2 M citric acid shaken 1 h RT	⟶	Exchangeable/ Carbonate
↓		
20 ml 0.04 M $NH_2OH.HCl$ in 25% HOAc at 96°C for 6 h	⟶	Reducible
↓		
20 ml 0.44 M NaOH 1 h and stirred	⟶	Organic
↓		
5 ml HF, 3 ml HNO_3, 3 ml HNO_3 sequential digestion	⟶	Residual

Table 58 : Fractionation of "4 metal" peat by the "Quick" Fractionation Scheme. Analysis by FAAS

	Concentration µg/g (DW)			
Fraction	Zn	Cd	Pb	Cu
Exch./Carbonate	289	134	5907	3948
Reducible	271	98	9927	2924
Organic	13	1	1698	449
Residual	240	1	4930	937
Total	813	234	22462	8258
Acid digest (HF/HNO_3)	899	230	22412	8315

Table 59 : Fractionation of Orchard Leaves SRM 718 by the "Quick" Fractionation Scheme. Analysis by FAAS

	Concentration µg/g (DW)			
Fraction	Zn	Cd	Pb	Cu
Exch./Carbonate	7.8	N.D.	7.1	2.9
Reducible	9.0	N.D.	4.7	2.4
Organic	6.4	N.D.	7.8	2.0
Residual	5.6	N.D.	15.1	4.2
Total	28.8	N.D.	34.7	11.5
Acid digest (HF/HNO_3)	17.8	N.D.	42.4	10.4
NBS Value	25.0	0.11	45.0	12.0

e) Apparatus and Instrumental Parameters

See Part II, Section 1 for the instrumental parameters used for FAAS and DPASV at an HMDE.

Analysis by DPASV at an RDE of heavy metals in prepared sample solutions

Apparatus :-

Model 174A	Polarograph Analyser (PARC)
Model 315A	Automated Electroanalysis Controller (PARC)
Model 628-10	Control/Power Unit, Metrohm.
Model 628-50	Drive Unit, Metrohm.
Model E554	Polarography Stand, Metrohm.
Model EA289/1	Glassy Carbon Disk Electrode, Metrohm.
Model EA880-20	Universal Titration/Polarography Vessel, Metrohm.
Model 7040A	X-Y Recorder, Hewlett Packard.

Method :-

The active surface of the glassy carbon electrode was renewed completely when it was used after long pauses or after contamination with undesired substances. Cleaning was achieved by polishing the electrode tip with α-aluminium oxide powder on a wet (DDW) polishing cloth (part of the Metrohm 6.2802.00 polishing kit). The polished front face was rinsed several times with DDW. The tip was then polished on the wet polishing cloth without any aluminium oxide powder. The front face was rinsed thoroughly.

The DPASV analyses were performed on 5 ml aliquots of the prepared sample in 15 ml of the required electrolyte buffer in a polarography vessel. A thin mercury-film electrode was deposited in situ with the analyte metals during the pre-electrolysis period, then stripped

completely during subsequent electrochemical conditioning. The mercury was supplied as a 40 µl aliquot of 5×10^{-2} M mercuric nitrate. The Model 628-10 Control/Power Unit was switched on at the cycle start and the glassy carbon electrode was rotated at the required speed, until the equilibration step.

Calibration was carried out by the method of standard additions, in all cases. Each addition was of 20 µl of a new standard metal solution (10 µg/ml). The metal content of the sample was obtained by extrapolation and the concentration estimated.

Instrumental Conditions

Model 315A

Conditioning Potential	0.0 V	vs. SCE
Initial Potential	-0.85 V	vs. SCE
Final Potential	-0.05 V	vs. SCE
Purge Time	10 min	
Conditioning Time	3 min	
Equilibrium Time	30 s	
Deposition Time		

Model 174A

Scan Rate	5 mV/s
Scan Direction	"+"
Modulation Amplitude	25 mV
Current Range	0.5 mA
Drop Time	0.05 s
Display Direction	"-"
Low Pass Filter	OFF

Mode	Differential Pulse
Initial Potential	0.0
Potential Scan Range	1.5 V

Model 628-10

Rate	500 rpm

A diagram of the Rotating Disk Electrode is given in Figure 41.

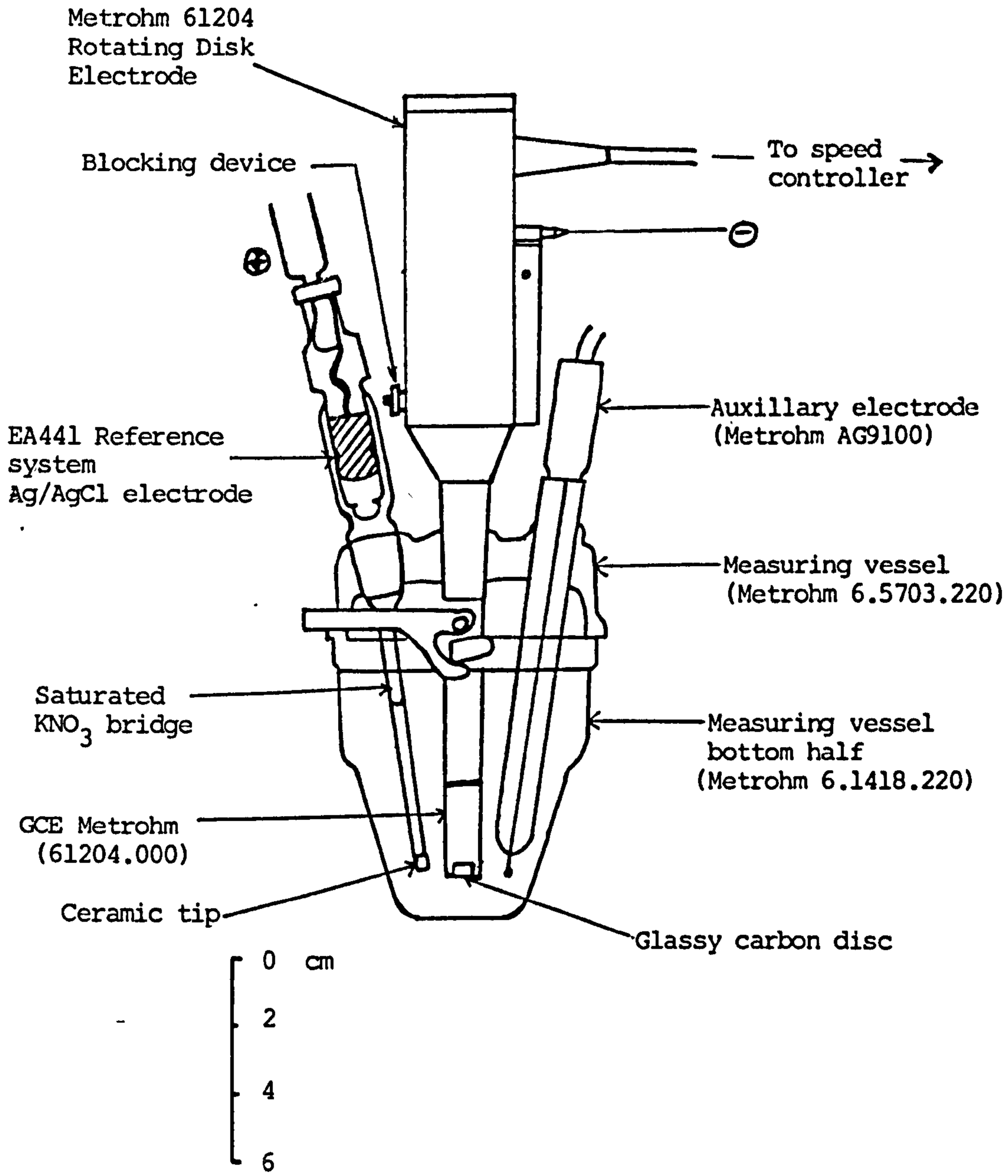


Figure 41 : Metrohm 628 Rotating Disk Electrode with glassy carbon tip

SECTION 4 : HUMIC AND FULVIC ACIDS : INTERACTIONS WITH HEAVY METALS

4.1 Experimental Preparation

a) Reagents

b) Extraction procedure

c) Purification procedure

i) humic acid

ii) fulvic acid

d) Analysis and Results

e) Apparatus and Instrumental Parameters

SECTION 4 : HUMIC AND FULVIC ACIDS : INTERACTIONS WITH HEAVY METALS

a) Reagents

AristaR hydrochloric and hydrofluoric acids.

AnalaR sodium hydroxide, sodium tetrapyrophosphate, ammonium acetate.

Aldrich Chemical Co. Ltd., Humic acid, sodium salt (mp > 300°C), technical grade.

b) Extraction Procedure

Both physical and chemical methods of extraction were applied to Levington's Universal Mixture (soil-less mixture).

1) Physical

Approximately 10 g of Levington's Mixture was sonicated in 200 ml of DDW, for 1 hour. The solution was filtered through Whatman 541 filter paper. Aliquots of the filtrate were added to a copper contaminated sample. Leaching of humic and fulvic acid from the Levington's Mixture.by this method was poor, therefore the chemical method of extraction suggested by Thorne (286) was used.

2) Chemical

Fulvic and humic acids were chemically extracted from 25 g of Levington's Mixture, according to the scheme outlined on pages 195 and 196. The dried soil-less mixture (25 g) was ground using a pestle and mortar, sieved through a 500μm mesh sieve and then poured into a 500 ml glass-stoppered conical flask. The extractant used was an equal volume mixture of 0.1 M NaOH and 0.1 M $Na_4P_2O_7$ (pH 13); 100 ml of this extractant mixture was added to the flask. A stream of oxygen-free, nitrogen gas was passed through the solution for 20 min, in order to prevent oxidation

Figure 42 : Chemical extraction of humic and fulvic acids, from Levington's Universal Mixture

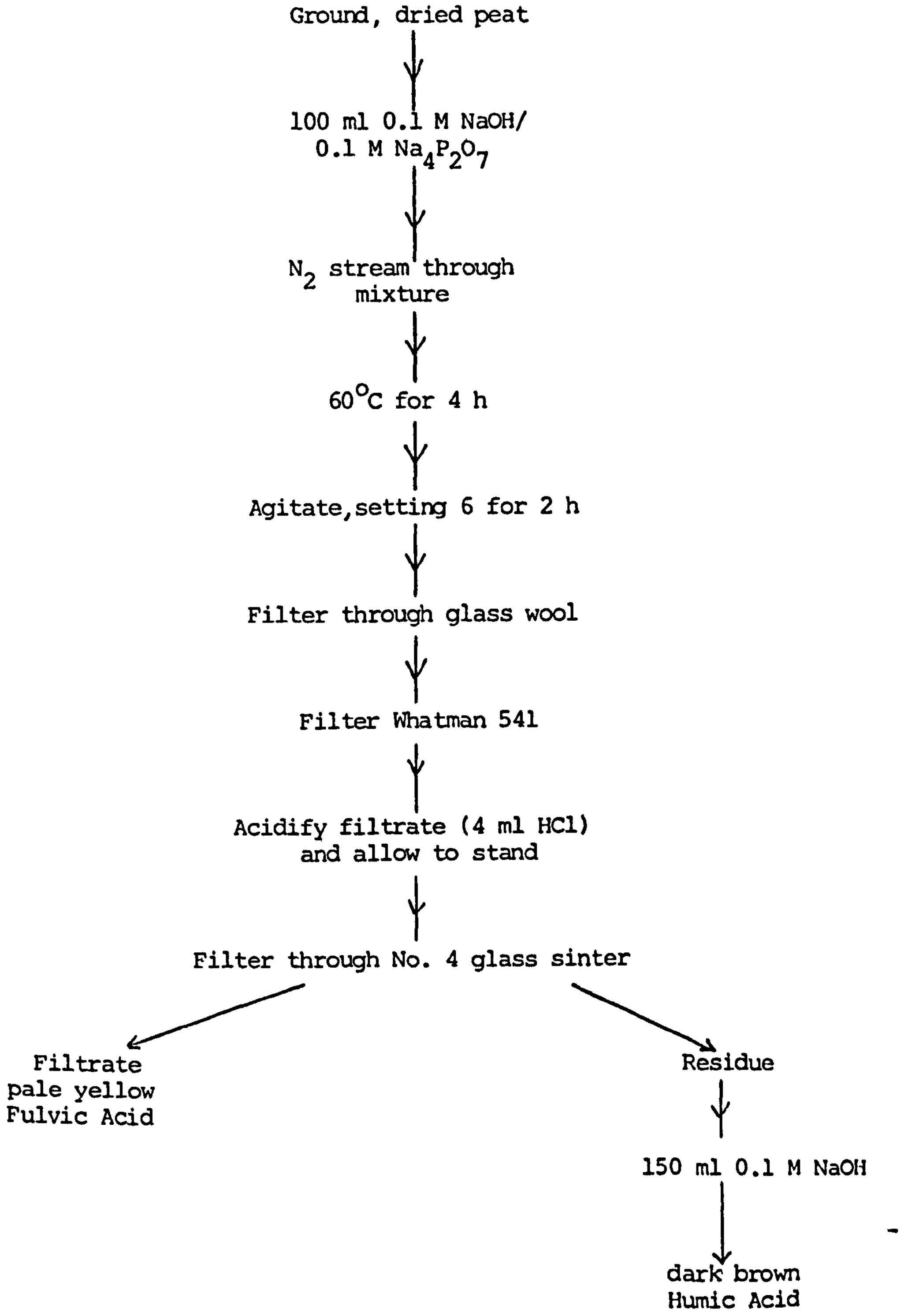
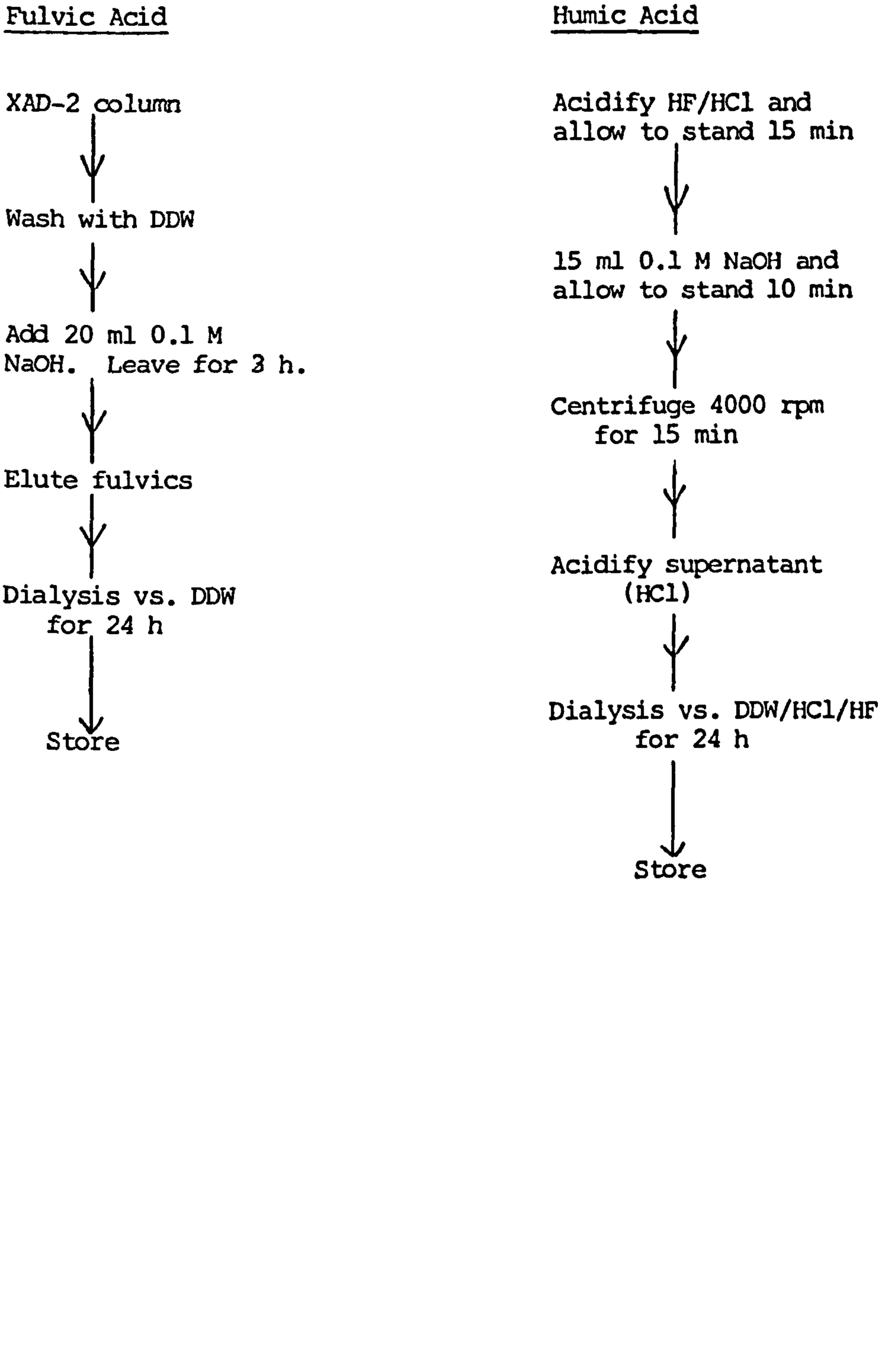


Figure 43 : Purification of humic and fulvic acids chemically extracted from Levington's Universal Mixture



of the organic matter constituents (287). The flask was placed in an oven at 60°C for 4 hours and then mechanically shaken (setting 6) for 2 hours. The mixture was filtered through three increasingly tightly-packed glass wool plugs, before being filtered under vacuum through a Whatman 541 filter paper on a Buchner funnel. The resultant filtrate (pH 9.0) was a clear, dark brown solution containing a mixture of humic and fulvic acids. The residue was humin (molecular weight > 10,000).

The filtrate was gently shaken and then allowed to stand. Concentrated hydrochloric acid (AristaR) (4 ml) was added to the solution. The mixture was shaken and allowed to stand. The solution, now a pale yellow, contained a grey-brown precipitate. The solution was left overnight, before being filtered through a No. 4 glass sinter, the pale yellow filtrate contained the fulvic acids. The residue containing the humic acid was redissolved in 150 ml of 0.1 M NaOH, to give a dark brown solution. Both humic and fulvic acids were purified.

c) Purification

1) Humic acid

The dark brown solution was acidified with a few drops of HF/HCl, and the mixture shaken and allowed to stand for 15 min. An aliquot (15 ml) of 0.1 M NaOH was added to the mixture and shaken, before being centrifuged at 4000 rpm for 15 min. The supernatant was decanted and acidified with sufficient HCl to cause precipitation. The precipitate thus formed was placed in a 40 cm length of Visking dialysis tubing (240 nm pore size). The humic acid was dialysed for 24 hours against DDW (acidified with three drops of HCl/HF). The humic acid was further dialysed against DDW until no chloride ion was detectable in the DDW. The precipitate was removed from the tubing and stored in an all glass stoppered flask.

2) Fulvic acid

The pale yellow solution was added to an XAD-2 column (length 15 cm; i.d. 1 cm). The column was washed with DDW. An aliquot (20 ml) of 0.1 M NaOH was added to the column and left for 3 hours, after which the fulvic acid was eluted, as two fractions F1 and F2. The fulvic acid was dialysed (40 cm of Visking dialysis tubing, pore size 240 nm) for 24 hours against DDW. The fulvic acid was removed from the tubing and stored in an all glass, stoppered flask.

c) Analysis and results

The concentration, ultra-violet spectrum and elemental composition of both fulvic and humic acid were determined. The results obtained are given in Tables 60 and 61 and Figure 44.

The extracted humic acid was examined at pH 7 (0.2 M ammonium acetate) by DPASV-HMDE, using the conditions given on page 150. Standard additions of 60 µl of 10 µg/ml Zn, Cd, Pb and Cu (no acid) were made. The voltammogram is given in Figure 45.

The extracted fulvic acids were also observed at pH 7, but using 20 µl standard additions of 10 µg/ml Zn, Cd, Pb and Cu (no acid). A typical voltammogram is given in Figure 45.

Aliquots (40 µl) of the extracted humic acid were added to a 10% solution of the Ystwyth sample Y_4 (< 5 µm, pH 7) and studied by DPASV-HMDE at pH 7 0.2 M ammonium acetate. The voltammogram is given in Figure 46. A graph of peak height vs. volume of humic acid added is given in Figure 47.

Aliquots (20 µl) of 10 µg/ml Cu solution (no acid) were added to a 2% v/v solution of the chemically extracted humic acid, and studied by DPASV-HMDE at pH 7 (0.2 M ammonium acetate). The voltammogram is given in Figure 48, and a graph of Cu peak height vs. Cu concentration is given in Figure 49. The procedure was repeated for Zn (5 µl aliquots)

Table 60 : Elemental Composition of Humic Acids

a) Humic acid chemically extracted from Levington's Universal Mixture

		Element				
		N	C	H	P	S
(i)	Percent	1.75	53.74	4.46	1.53	0.64
(ii)	Percent	0.86	53.79	4.65	-	0.60
	Mean %	1.31	53.77	4.56	-	0.62

Concentration of extracted humic acid = 3.64 mg/ml

b) Humic acid - Commercial preparation

		Element				
		N	C	H	P	S
(i)	Percent	0.50	48.45	4.35	-	-
(ii)	Percent	0.55	48.11	4.19	-	-
	Mean %	0.53	48.28	4.27	-	-

Table 61 : Elemental Composition of Fulvic Acid chemically extracted from Levington's Universal Mixture

a) Fl

		Element		
		N	C	H
(i)	Percent	1.80	32.34	2.98
(ii)	Percent	2.11	30.63	3.08
	Mean %	1.96	31.49	3.03

Concentration of Fl = 2.1 mg/ml

b) F2

		Element		
		N	C	H
(i)	Percent	1.49	30.87	2.97
(ii)	Percent	1.38	30.44	2.95
	Mean %	1.44	30.66	2.96

Concentration of F2 = 1.1 mg/ml

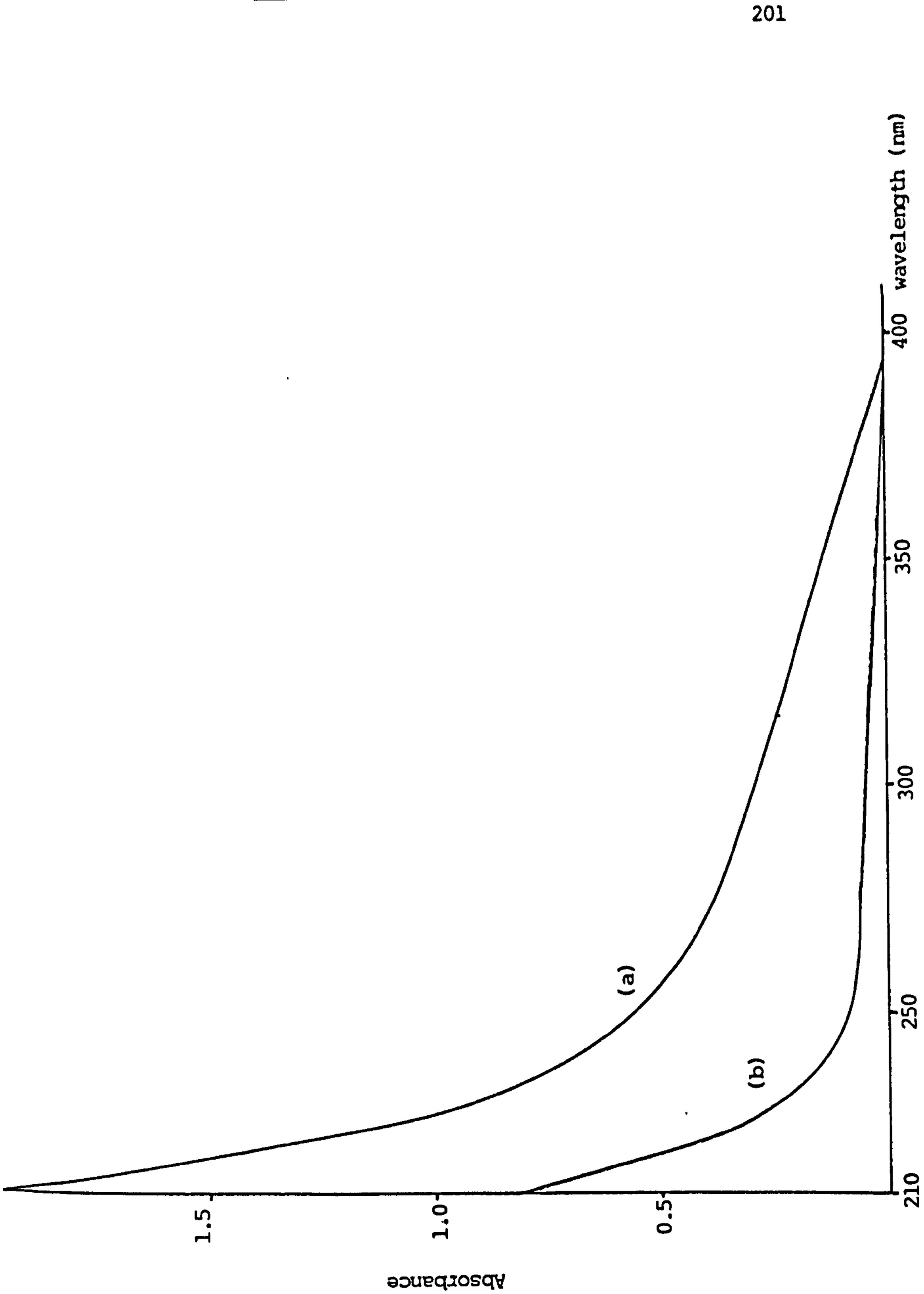


Figure 44 : UV Spectra of (a) Fulvic and (b) Humic Acids

Figure 45 : Differential pulse stripping curve (HMDE) of additions of Zn, Cd, Pb and Cu to a) FA solution, and b) HA solution, at pH 7

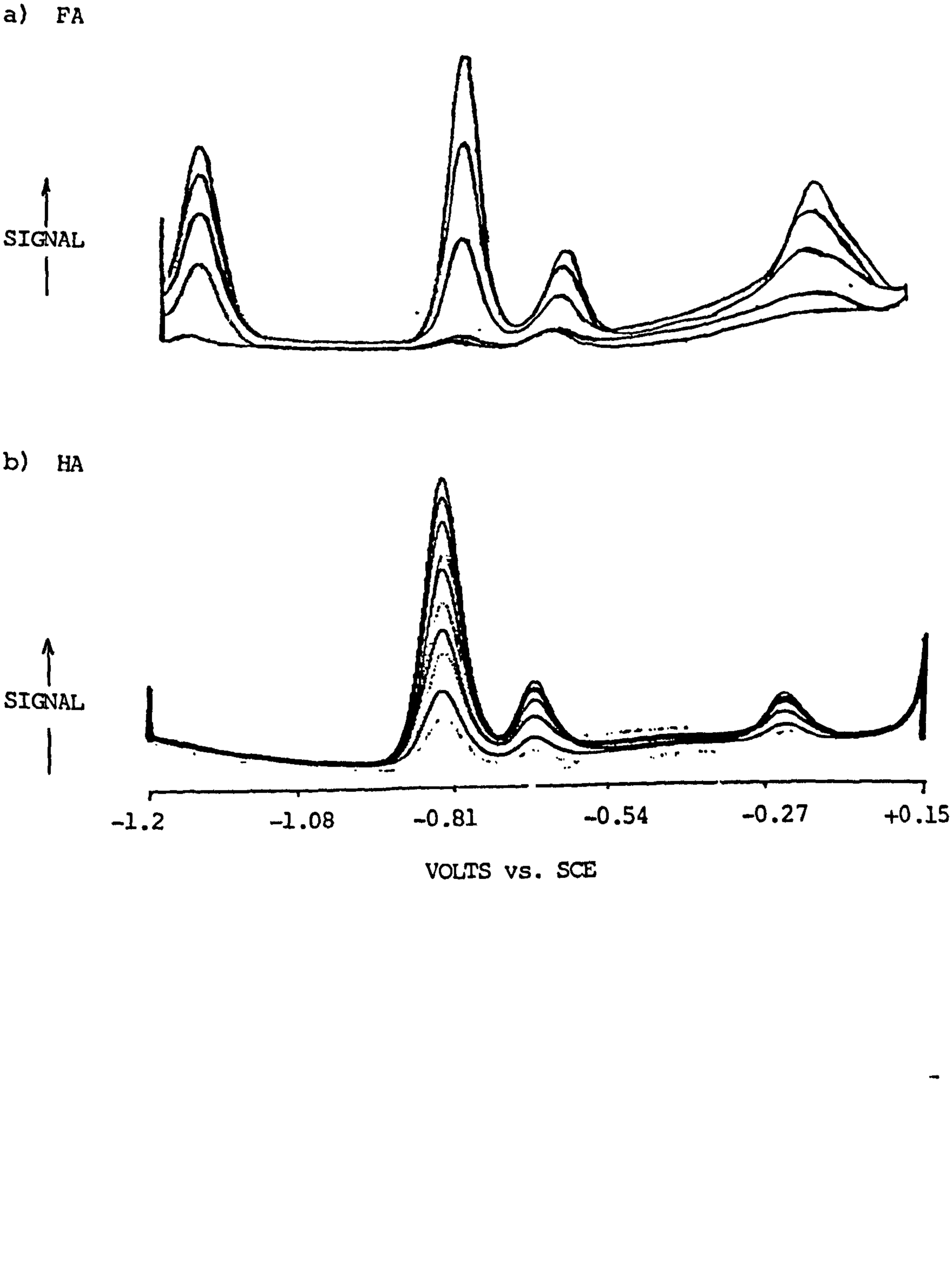


Figure 46 : Differential pulse stripping curve (HMDE) of the addition of HA to a solution of Zn (Y4 < 5 µm sample) at a pH of 7

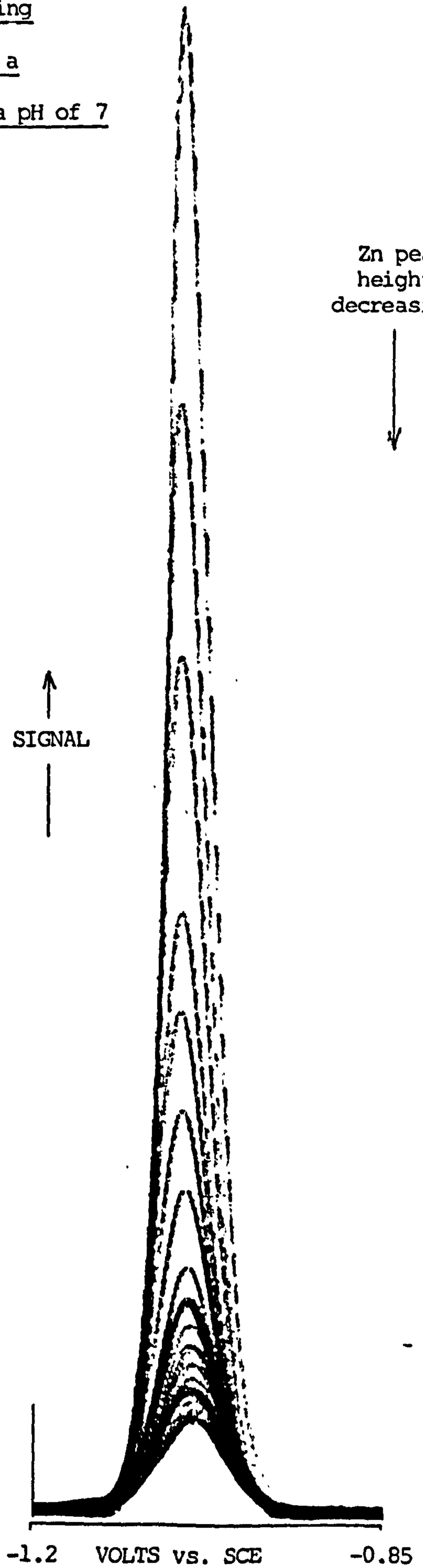


Figure 47 : Additions of humic acid (extracted from Levington's Universal Mixture) to a zinc contaminated sample (Ystwyth Y4 < 5 µu) at a pH of 7

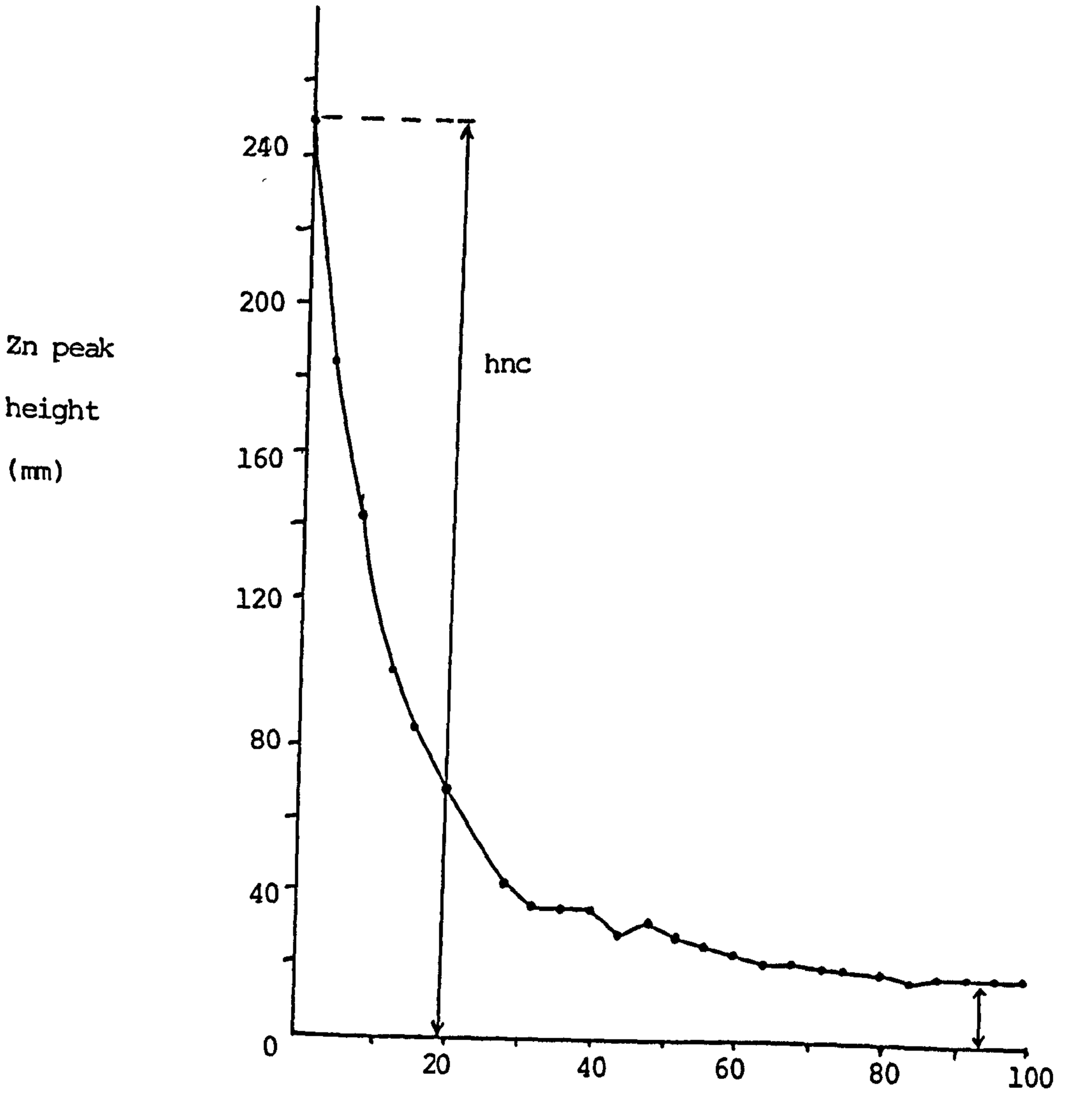


Figure 48 : Differential pulse stripping curve (HMDE) of the addition of Cu to a 2% v/v solution of HA at pH 7

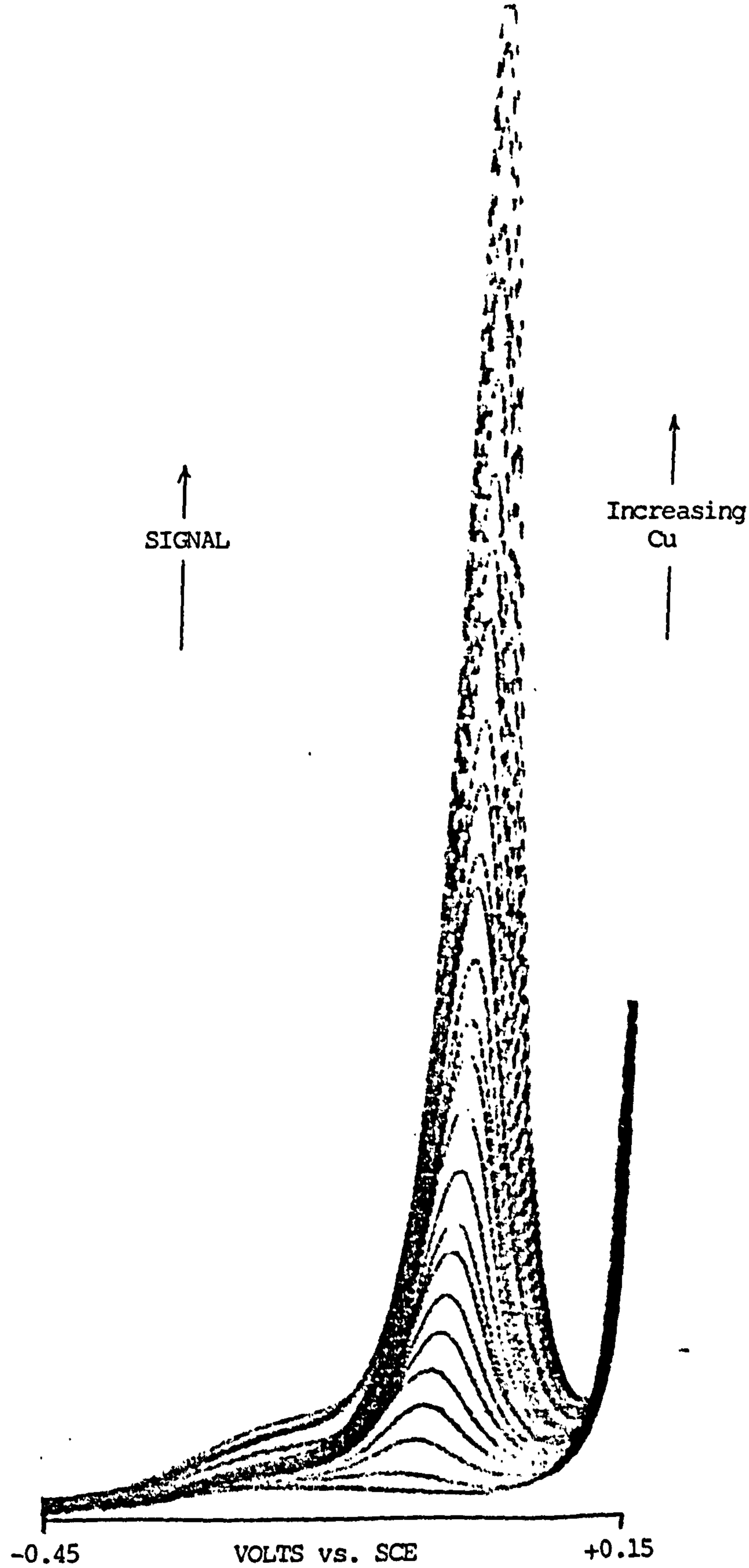
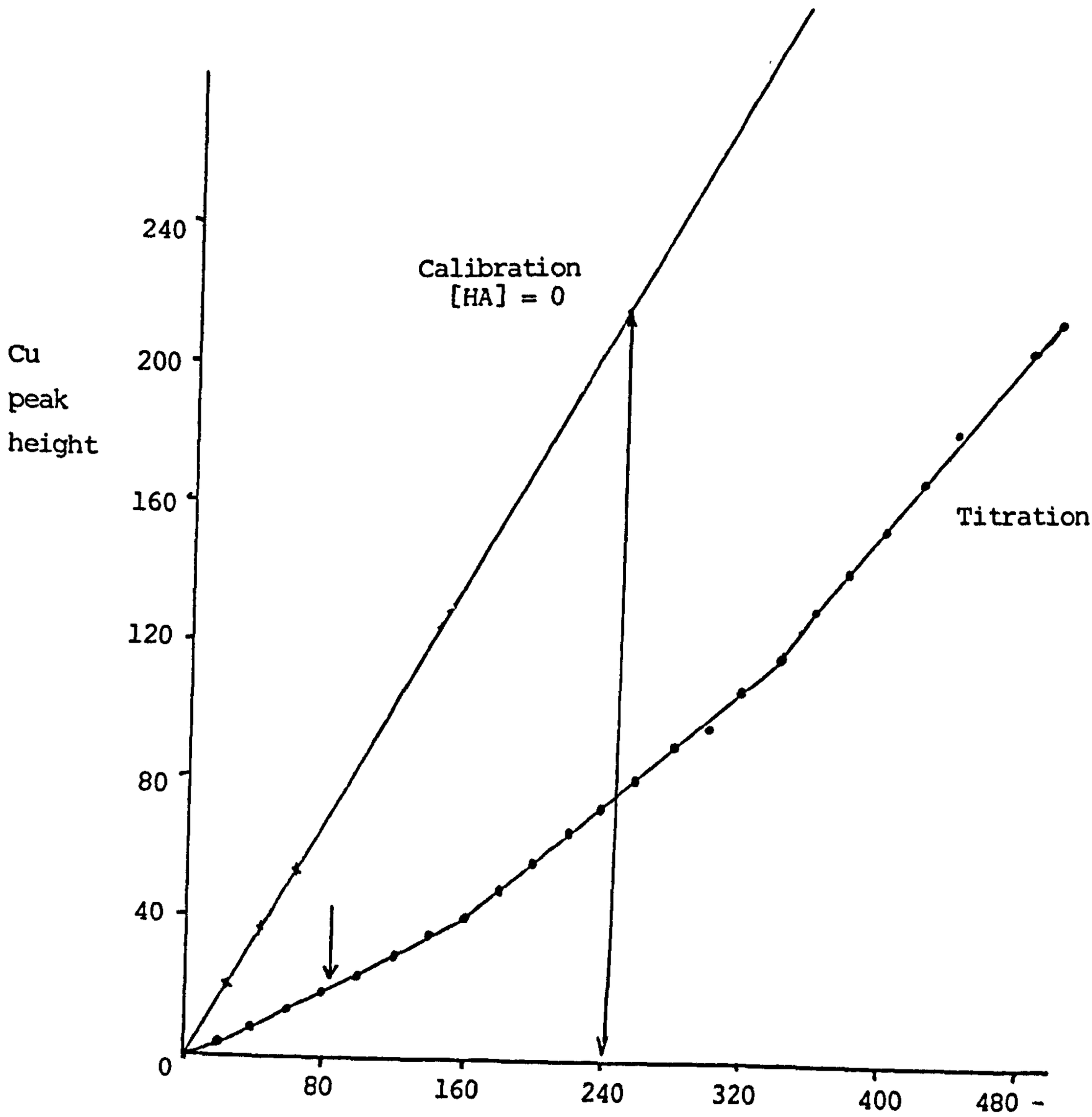


Figure 49: Additions of Cu (20 ng/ml) to 2% v/v Humic Acid at pH 7.

Cu peak height (mm) against Cu concentration (ng/ml)



Cd, (10 µl aliquots) and Pb (10 µl aliquots). The chemically extracted fulvic acid was similarly treated, using aliquots of Cu (40 µl), Zn (5 µl), Cd (20 µl) and Pb (5 µl) to 20% v/v solutions of the fulvic acid at pH 7. The voltammograms are given in Figures 50, 52, 54, 56, 58, 60 and 62 and graphs of peak height against metal concentration are given in Figures 51, 53, 55, 57, 59, 61,and 63.

Calibration graphs, with no ligand addition, were also prepared for each of the metals studied. Where possible the diffusion coefficients and stability constants for the metal-ligand complexes were determined.

Figure 50 : Differential stripping curve (HMDE) of the addition of Zn to a 2% v/v solution of HA at pH 7

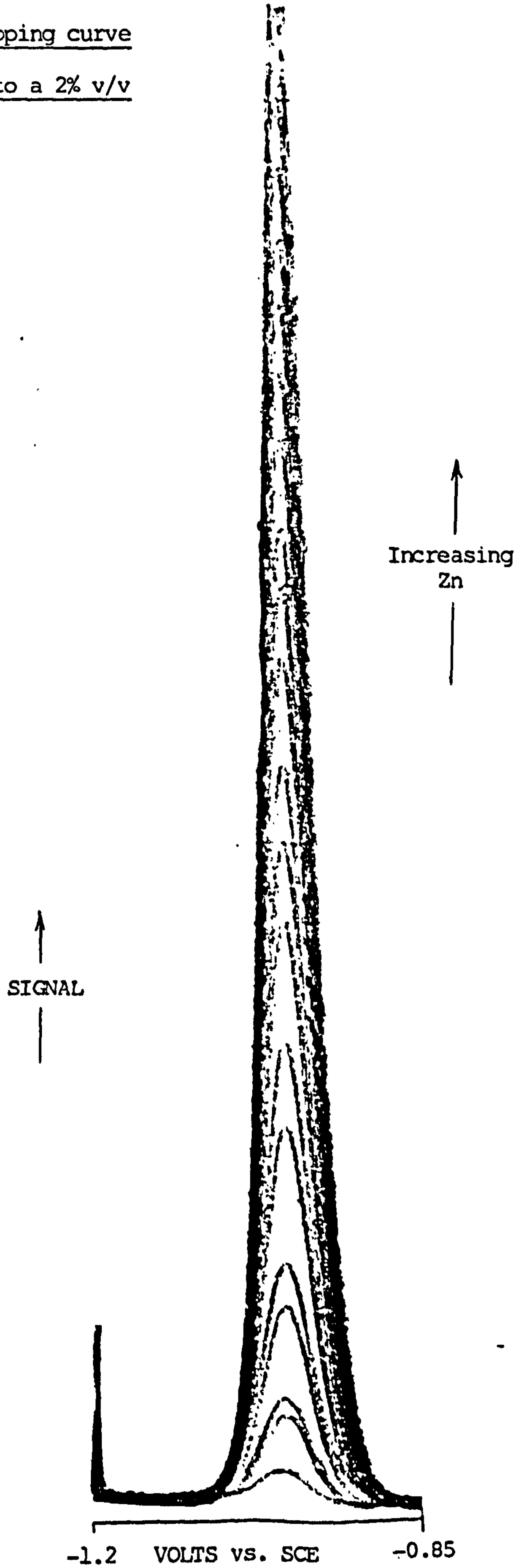


Figure 51 : Additions of Zn (5 ng/ml) to 2% v/v Humic Acid at pH 7.

Zn peak height (mm) vs. Zn concentration (ng/ml)

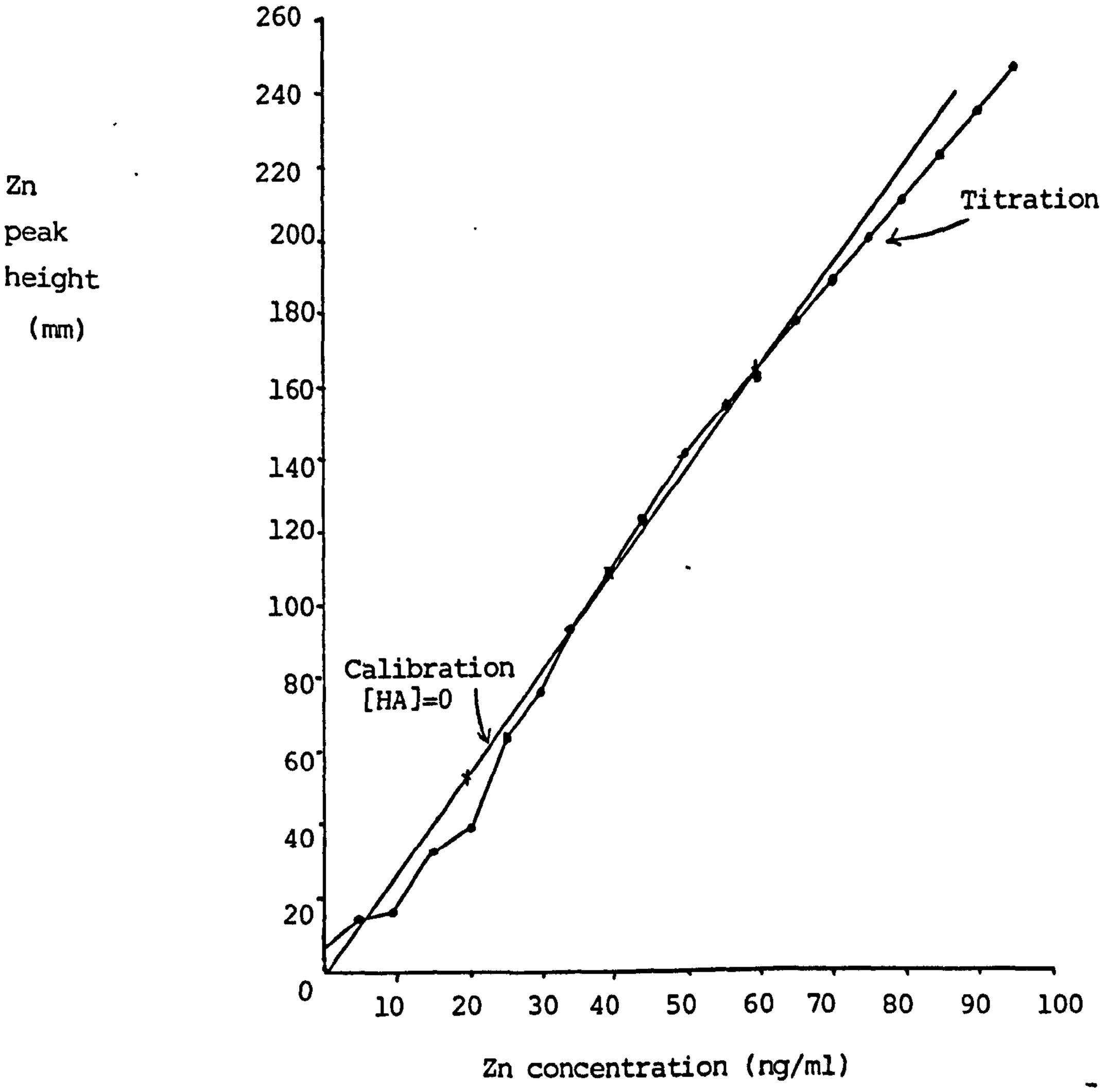


Figure 52 : Differential pulse stripping curve (HMDE) of the addition of Cd to a 2% v/v solution of HA at pH 7

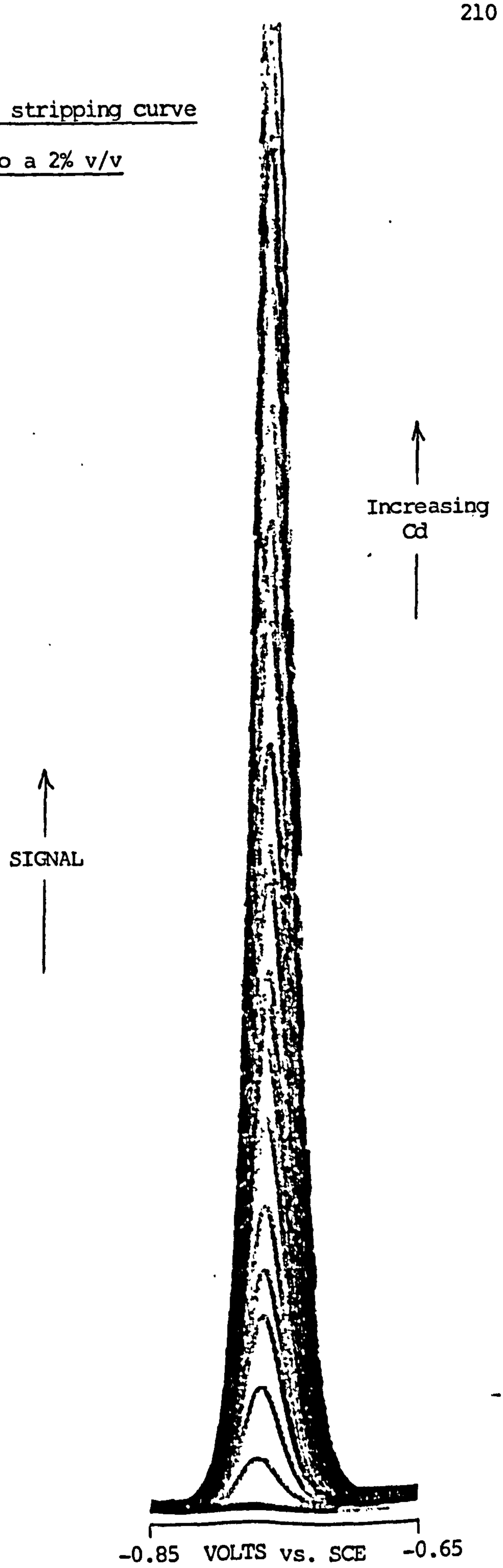


Figure 53 : Additions of 20 ng/ml Cd to 2% v/v Humic Acid at pH 7.

Cd peak height (mm) vs. Cd concentration (ng/ml)

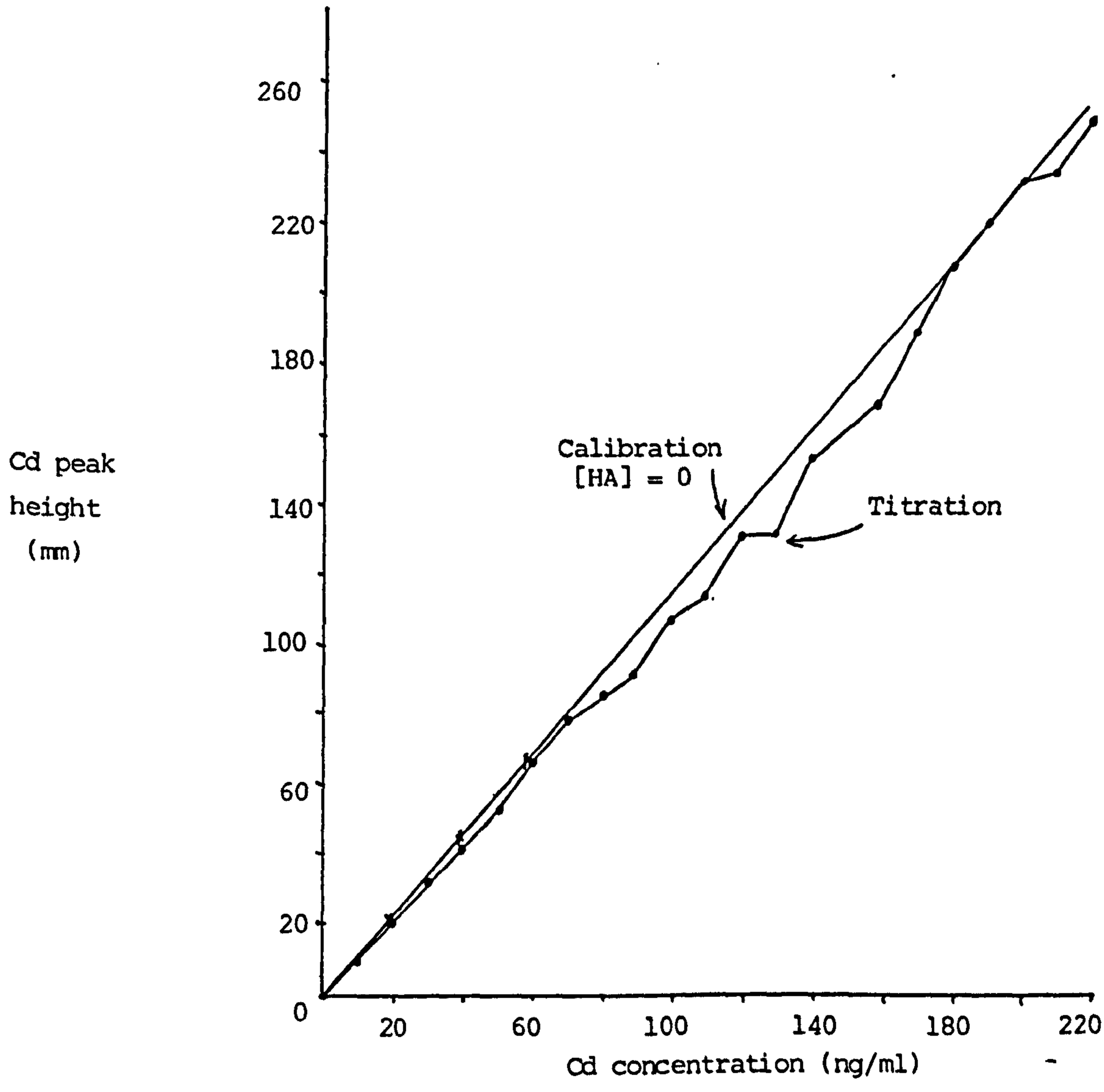


Figure 54 : Differential pulse stripping curve (HMDE) of the addition of Pb to a 2% v/v solution of HA at pH 7

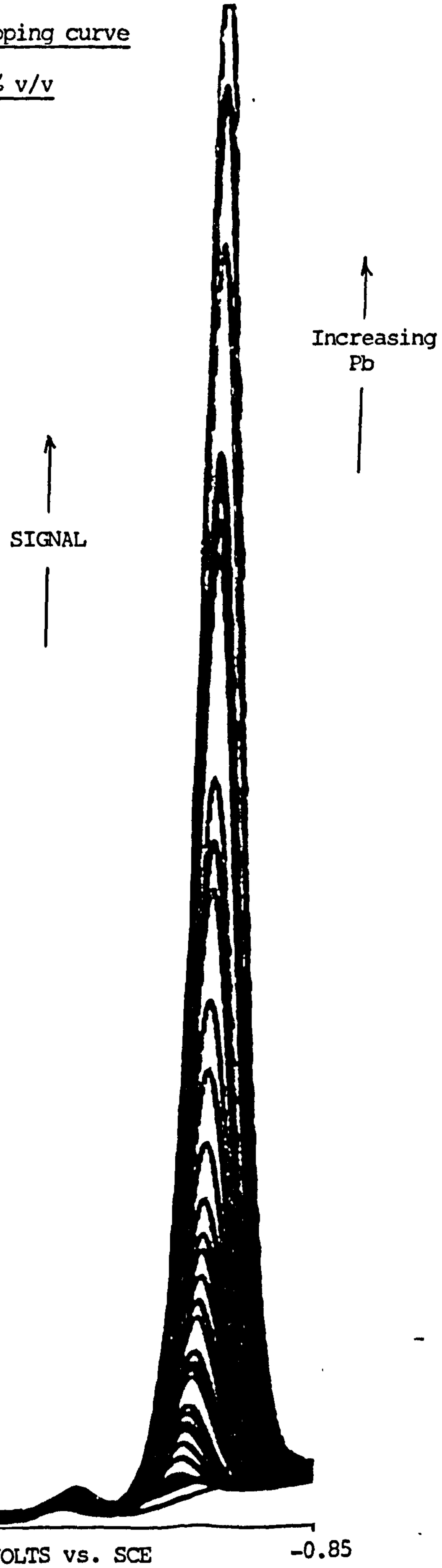


Figure 55 : Additions of Pb (10 ng/ml) to 2% v/v Humic Acid at pH 7.

Pb peak height (mm) vs. Pb concentration (ng/ml)

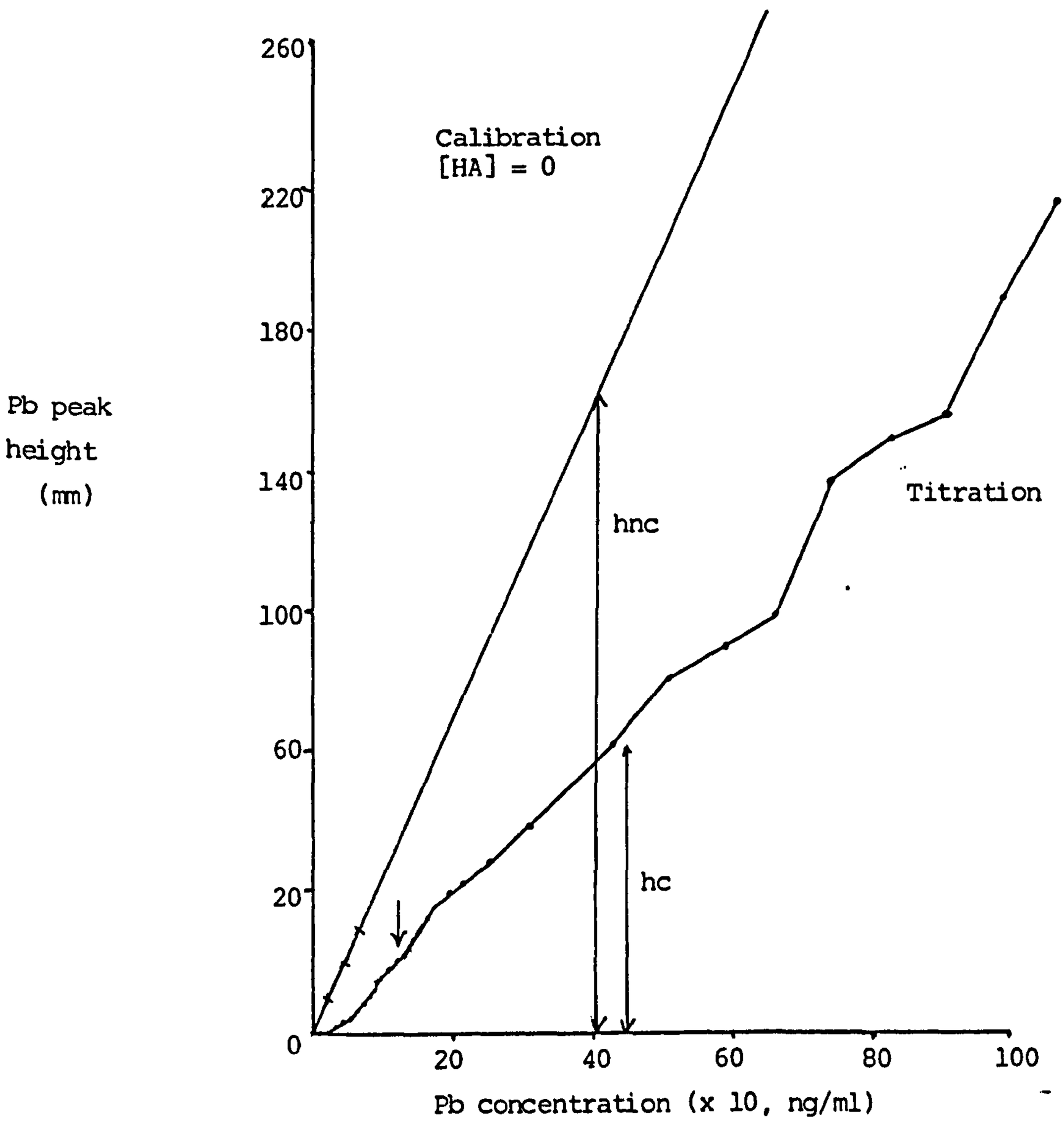


Figure 56 : Differential pulse stripping curve (HMDE) of the addition of Cu to a 2% v/v solution of FA at pH 7

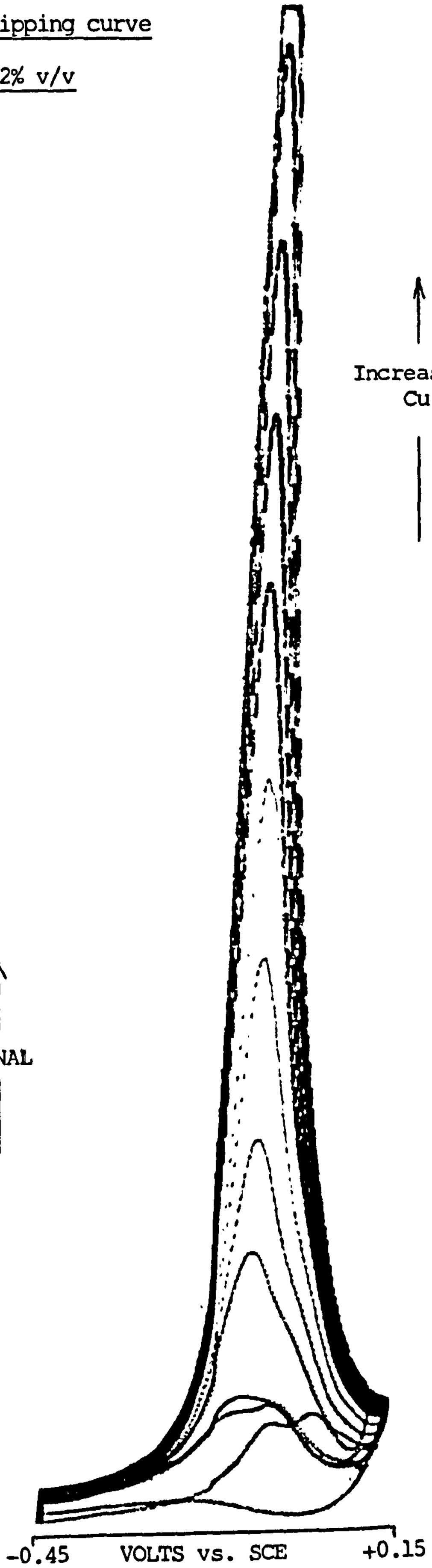


Figure 57 : Additions of Cu (40 ng/ml) to 20% v/v Fulvic Acid.

Cu peak height (mm) vs. Cu concentration (ng/ml)

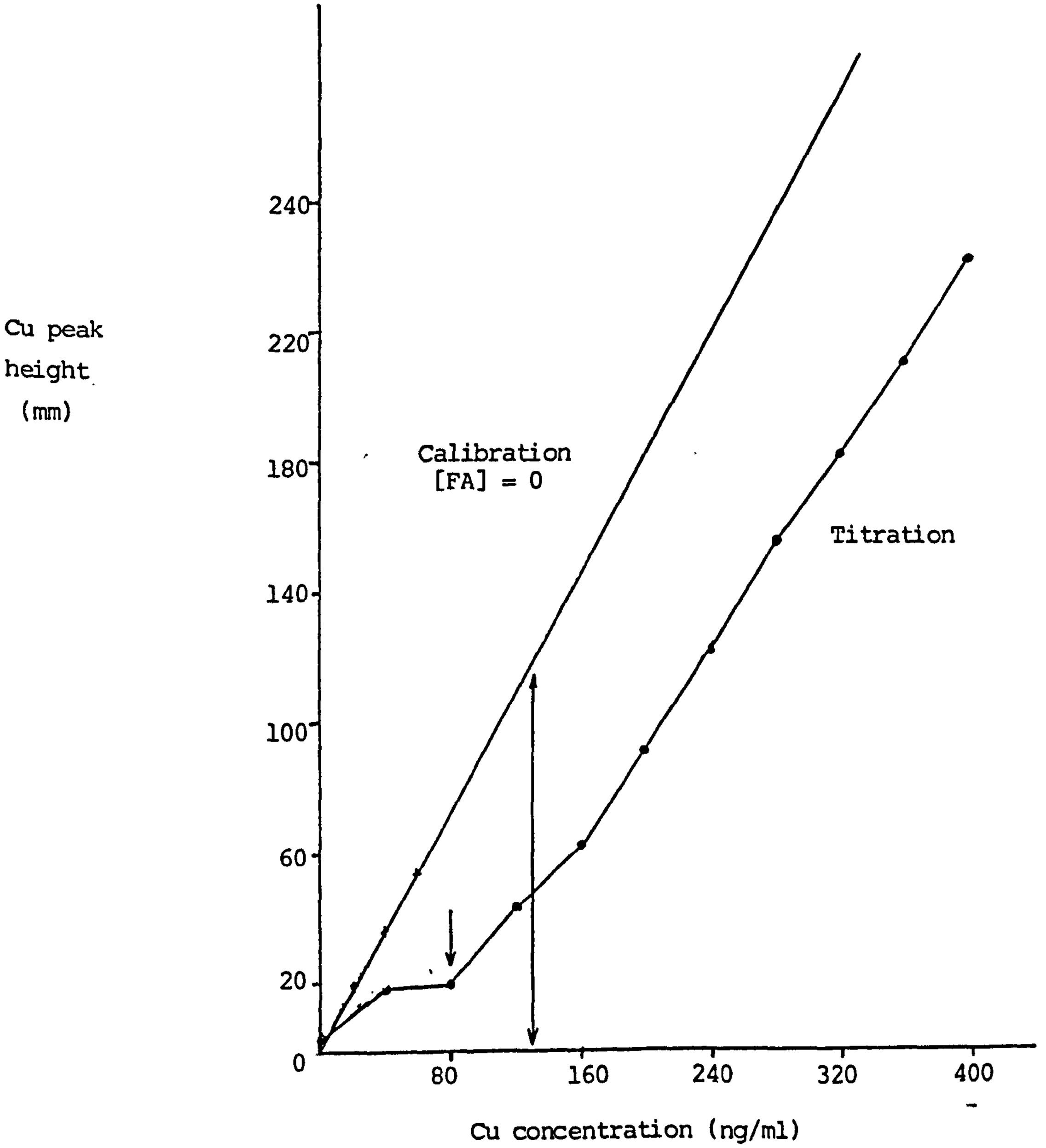


Figure 58 : Differential pulse stripping curve (HMDE) of the addition of Zn to 20% v/v FA solution at pH 7

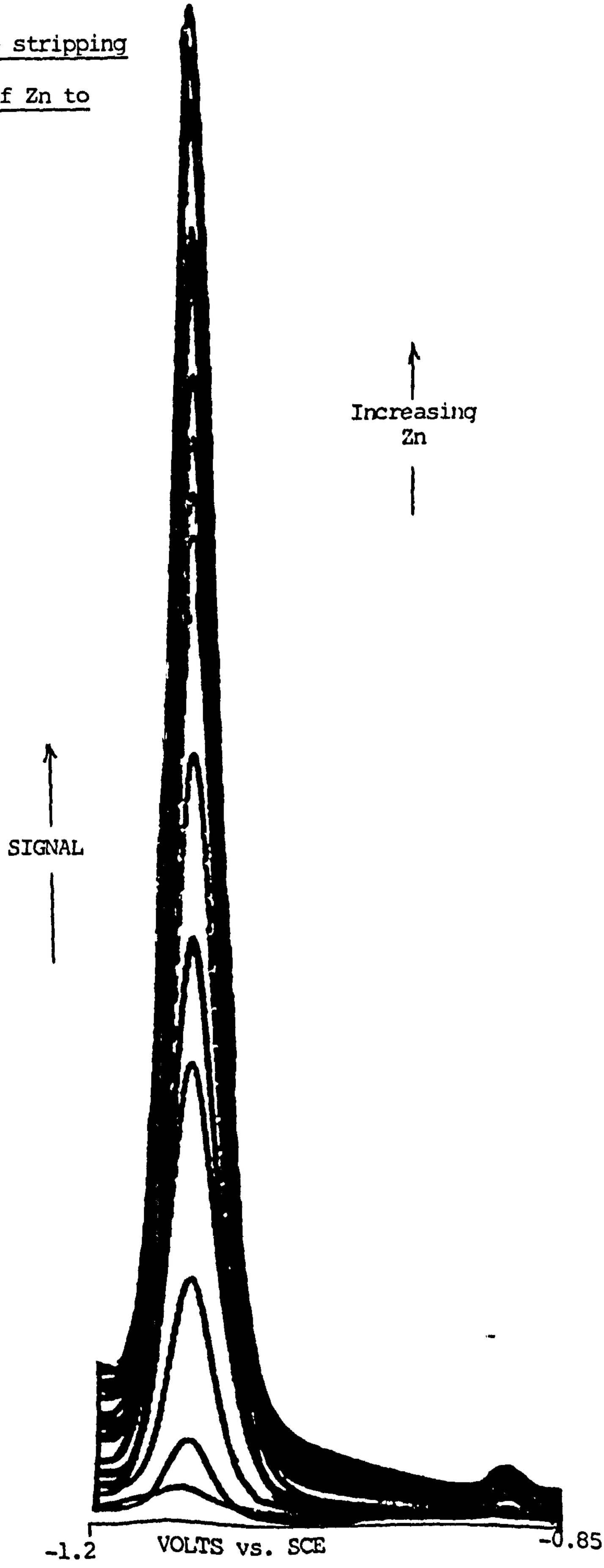


Figure 59 : Additions of 20 ng/ml Zn to 20% v/v Fulvic Acid.

Zn peak height vs. Zn concentration (ng/ml)

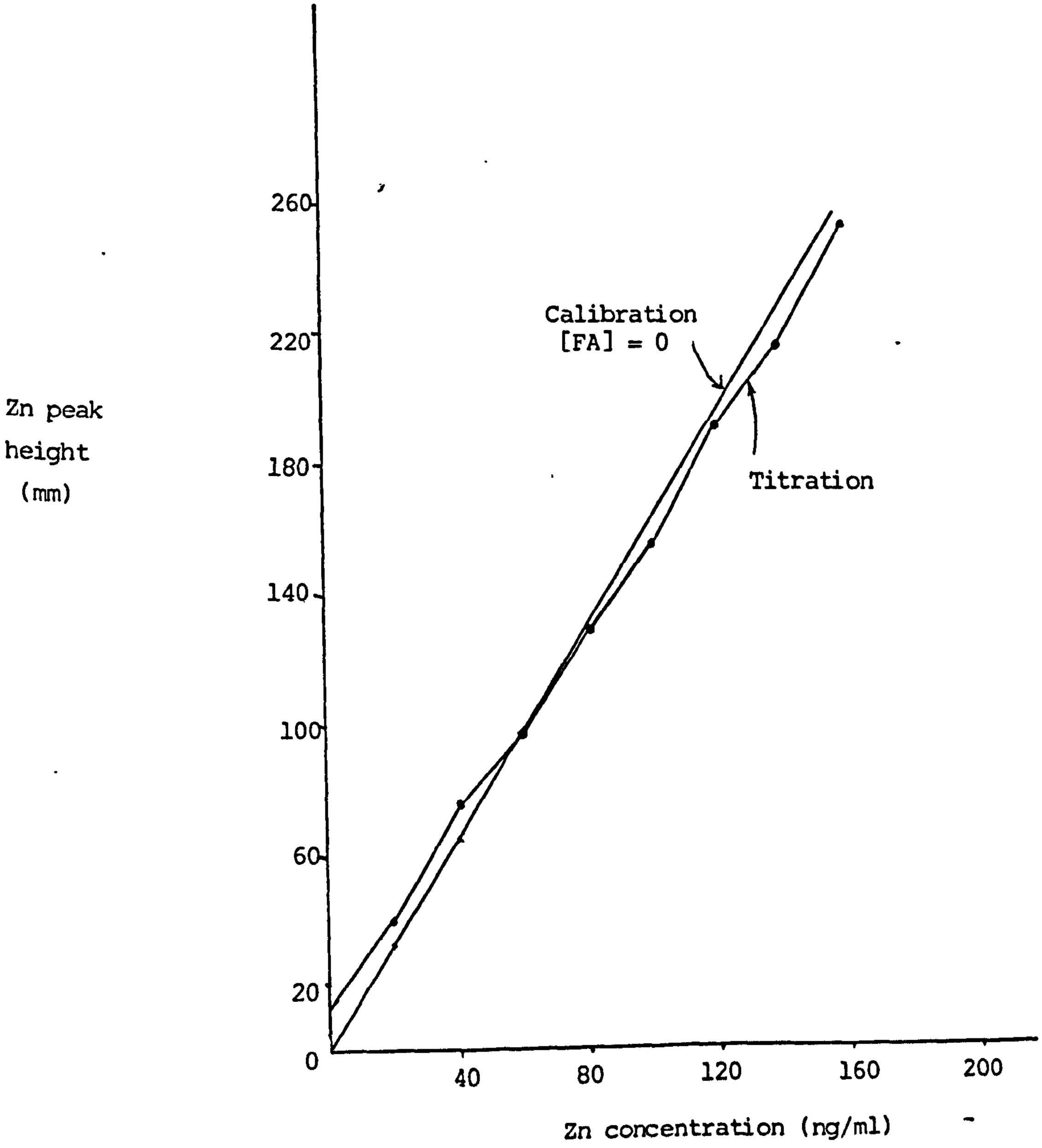


Figure 60 : Differential pulse stripping curve (HMDE) of the additions of Cd to 20% v/v FA, at pH 7

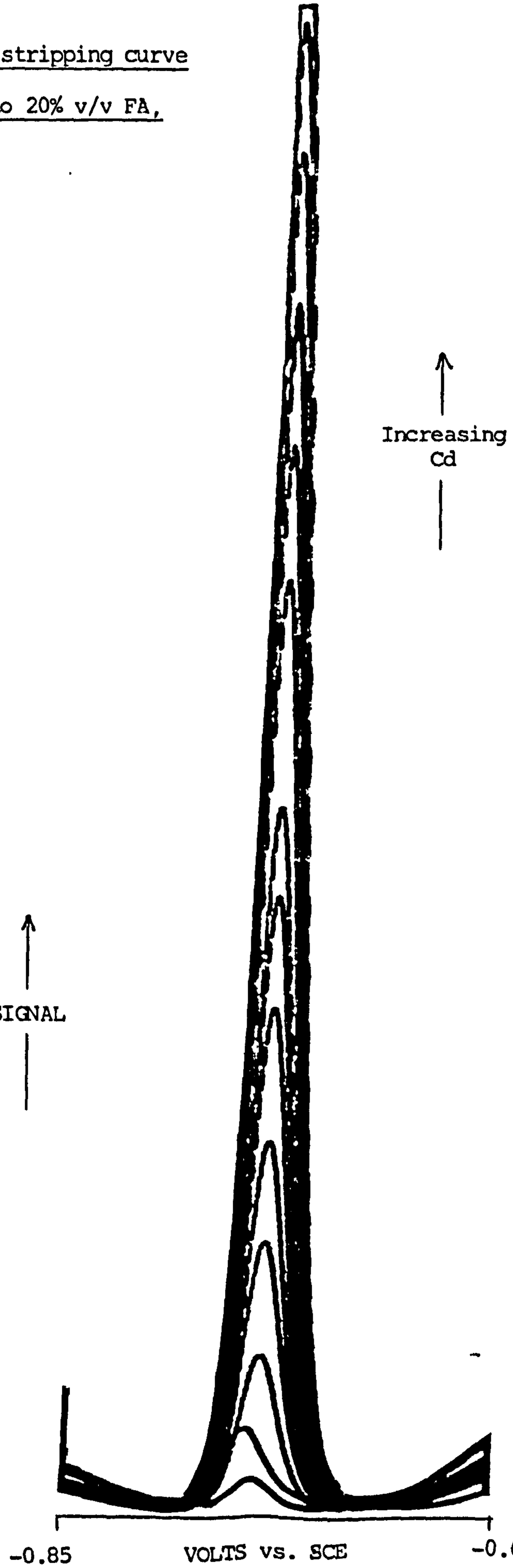


Figure 61 : Additions of Cd (20 ng/ml) to 20% v/v Fulvic Acid at pH 7.

Cd peak height (mm) vs. Cd concentration (ng/ml)

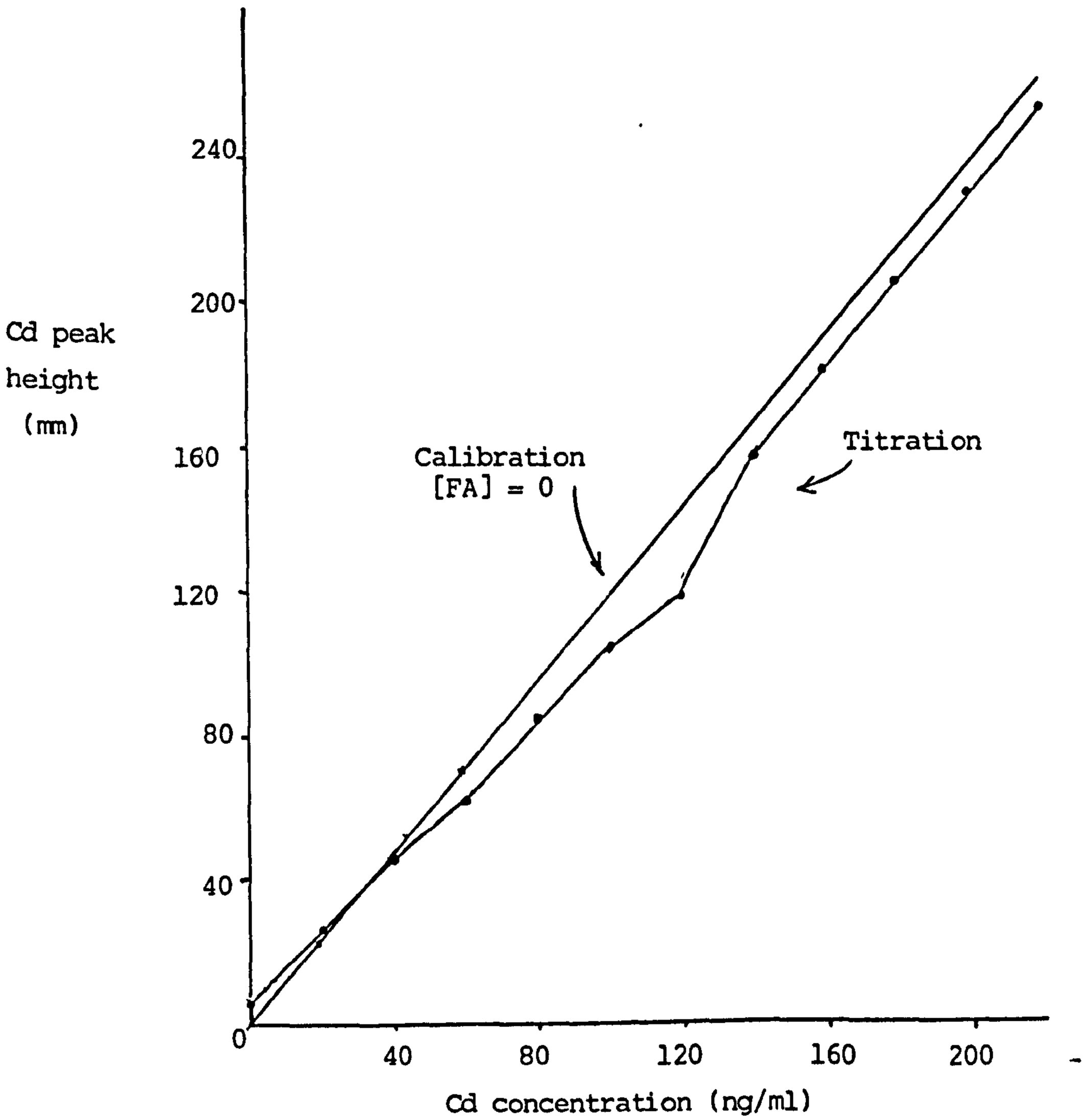


Figure 62 : Differential pulse stripping curve (HMDE) of the addition of Pb to 20% v/v FA at pH 7

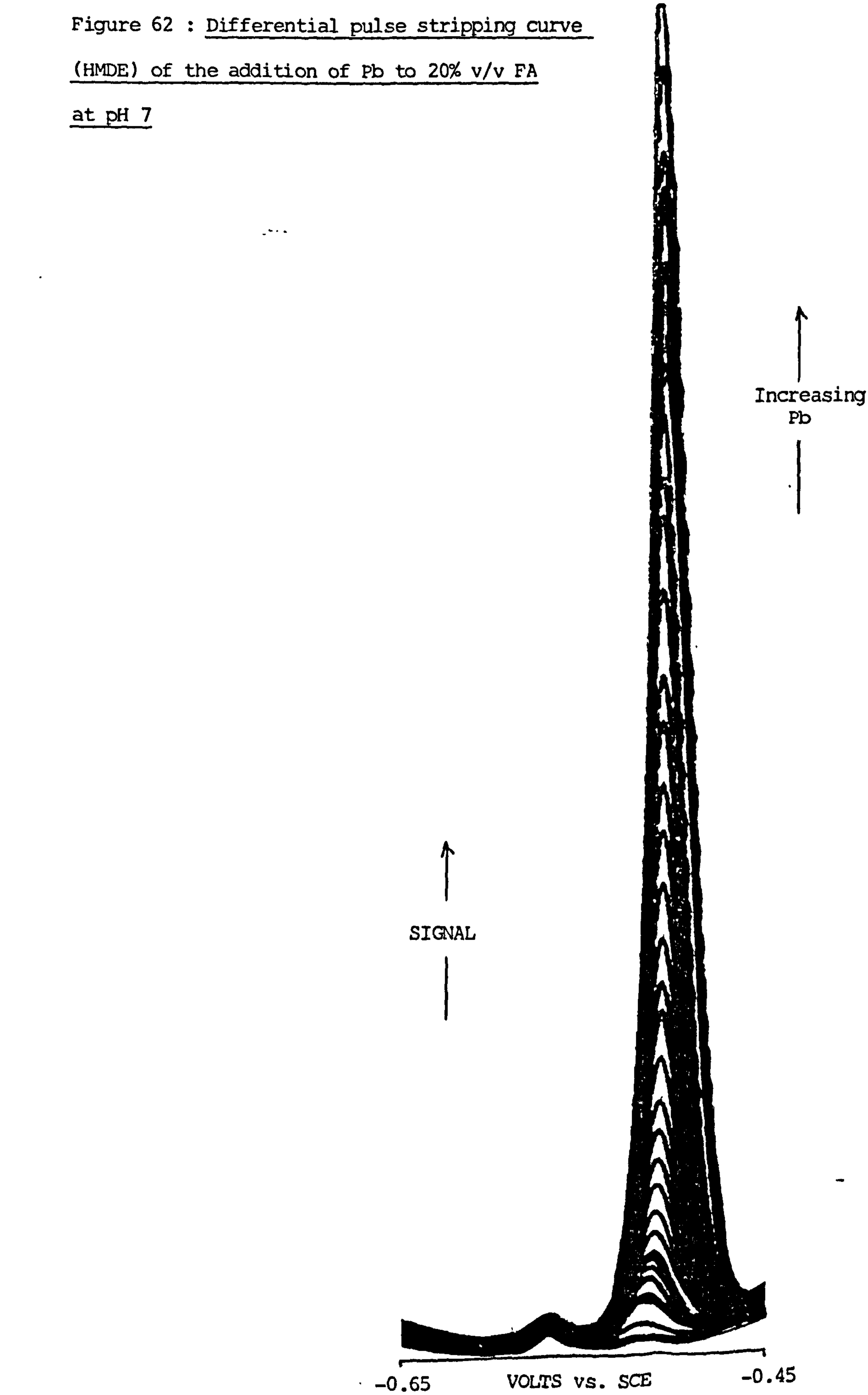
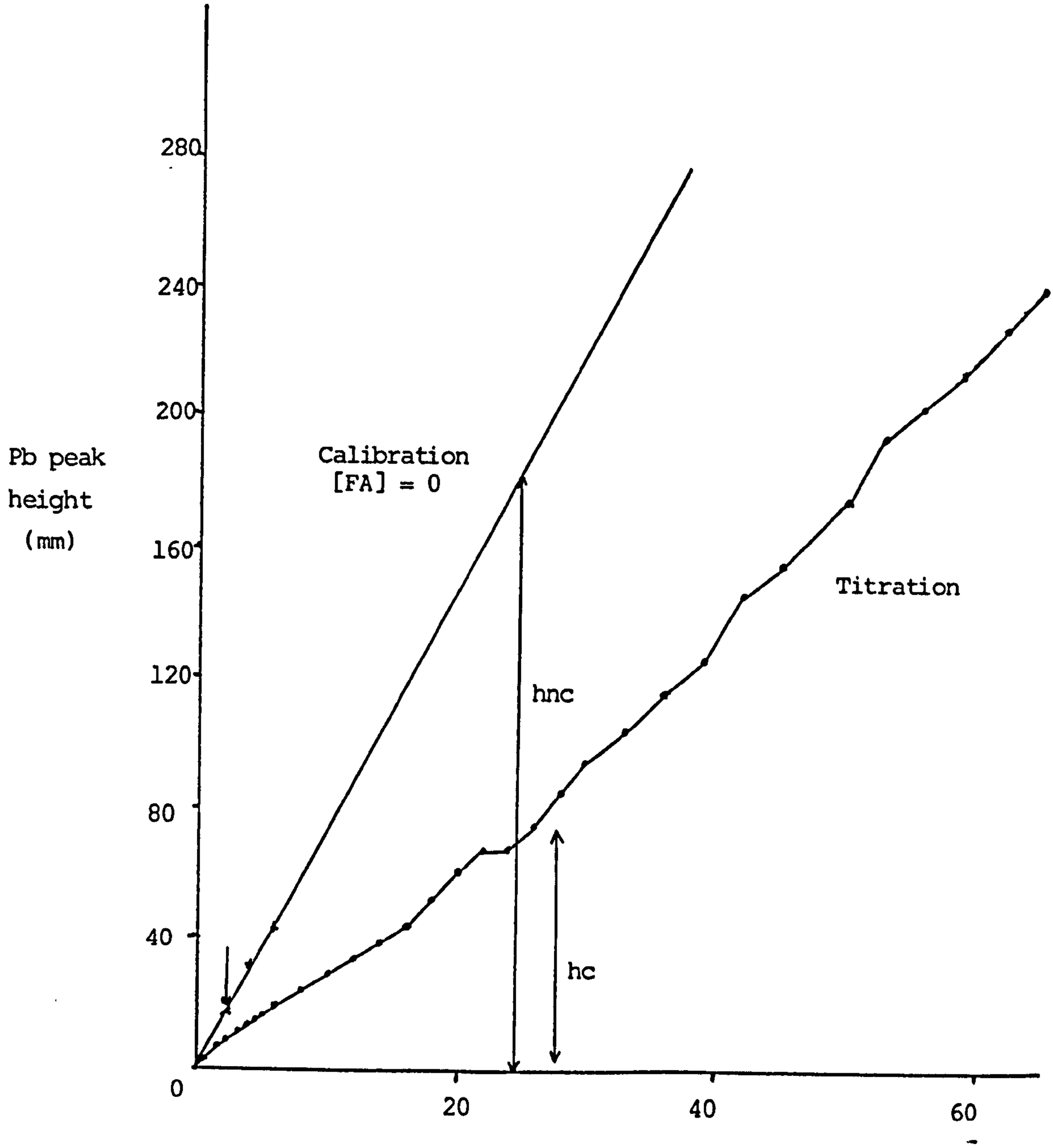


Figure 63 : Additions (5 ng/ml) of Pb to 20% v/v Fulvic Acid at pH 7.

Pb peak height (mm) vs. Pb concentration (x 10 ng/ml)



The current (which is proportional to peak height) in the stripping process is related to the concentration of metal in the mercury drop. The concentration of metal in the HMDE, accumulated during the deposition process, is in turn proportional to the diffusion coefficient to the two-thirds power, i.e.

$$\text{peak height} \propto \text{current} \propto \text{concentration in Hg drop} \propto D^{\frac{2}{3}}$$

$$\left(\frac{Dnc}{Dc}\right)^{\frac{2}{3}} = \frac{hnc}{hc}$$

where Dnc is the diffusion coefficient for the non-complexed species, and Dc is the diffusion coefficient for the complexed species, hnc is the peak height for the non-complexed species and hc the peak height for the complexed species.

$$Dc = Dnc. \left(\frac{hc}{hnc}\right)^{3/2}$$

Substituting values for Cu-Ha

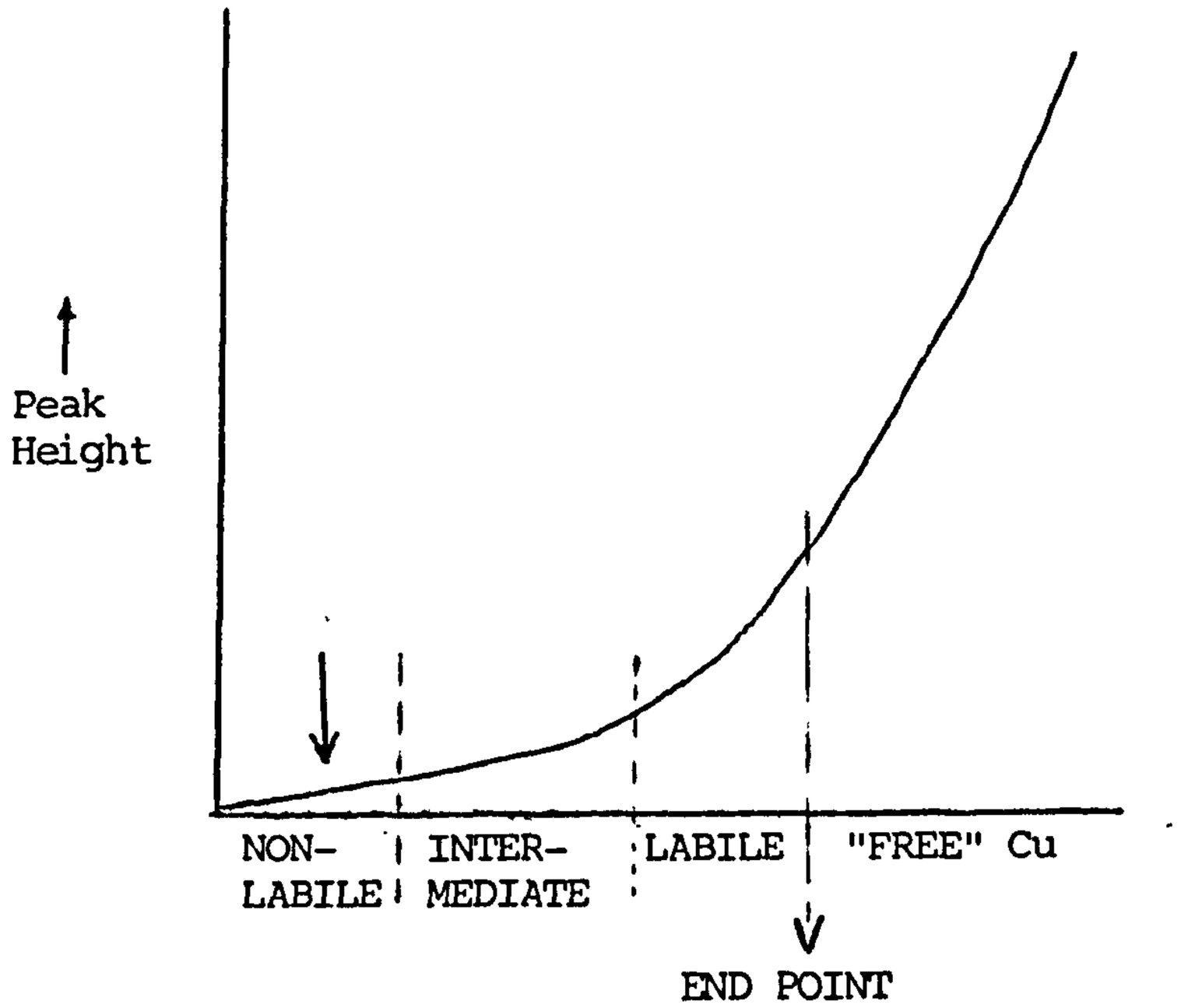
$$\text{Dnc for } Cu^{2+} = 7.2 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1} \quad (288)$$

$$Dc = 7.2 \times 10^{-6} . \left(\frac{3.7}{10.8}\right)^{3/2}$$

$$= \underline{1.4 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}}$$

From Figure 49 it can be seen that the titration curve may be partitioned as follows :

Figure 49a : Detailed Titration Curve of HA by Cu using DPASV



Having assumed the non-lability of the complex at high HA : Cu ratio, the stability constant may be calculated, citing the peak height as representative of the concentration of Cu^{2+} at equilibrium, i.e. :-

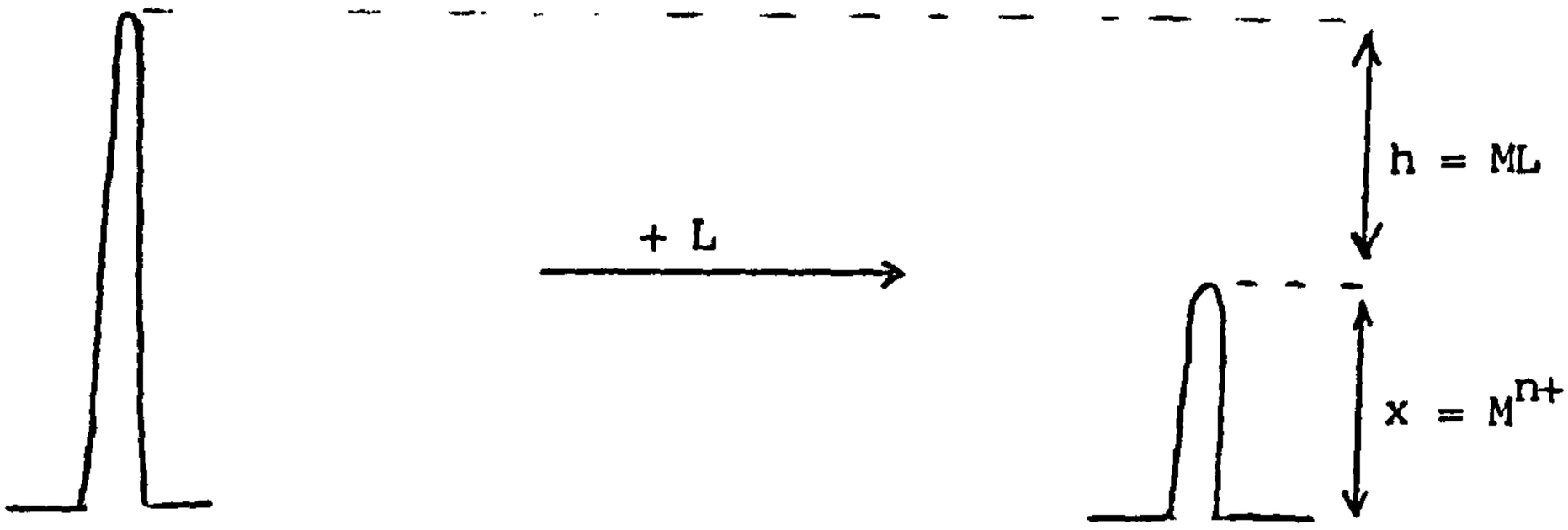
$$M^{n+} + L \rightleftharpoons ML^{n+}$$

where M^{n+} is the metal and L is the ligand.

$$K_{ML} = \frac{[ML^{n+}]}{[M^{n+}][L]}$$

An estimation of the stability constant is often made by measuring the reduction of peak height (representing the concentration of metal in solution) following the addition of a complexing agent, and citing the

difference as the amount of metal bound by the ligand :-



ASV peak height before addition of ligand

ASV peak height after addition of ligand

Height h represents the fraction of metal bound to the ligand, height x represents "free" (or hydrated) metal ion.

A point on the curve (non-labile section of Figure 49a ; arrowed) of height 1 cm was taken and the concentration determined - 84 ng/ml i.e. 1.32×10^{-6} M.

From the calibration curve (i.e. no ligand present) 1×10^{-6} M was represented by a height of 0.06 cm. Thus, 1 cm represents $\frac{1}{0.06} \times 10^{-6}$ M = 1.67×10^{-7} M.

[CuHA] = Actual Cu concentration added - Cu concentration observed (i.e. remaining non-complexed)

= $1.32 \times 10^{-6} - 1.67 \times 10^{-7} = 1.15 \times 10^{-6}$ M

Assuming a 1:1 complex was formed and that the molecular weight of HA is approximately 4000, then :-

[HA] = HA concentration added - complexed Cu concentration

= $8.75 \times 10^{-6} - 1.15 \times 10^{-6}$

= 7.6×10^{-6} M.

Substituting directly into the formula :-

$$K_{ML} = \frac{[ML^n]}{[M^{n+}][L]}$$

$$K = \frac{1.15 \times 10^{-6}}{(1.67 \times 10^{-7}) \times (7.6 \times 10^{-6})}$$

$$K = \underline{9 \times 10^5 \text{ mol}^{-1}\text{l}}$$

(a conditional stability constant)

The procedure was applied to the other metal-HA and metal-FA reactions :-

For Cu-FA

$$Dc = 7.2 \times 10^{-6} \cdot \left(\frac{2.4}{5.85}\right)^{3/2} = \underline{1.89 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}}$$

Assuming a molecular weight of 650 and a 1:1 complex

$$K = \frac{9.15 \times 10^{-7}}{(3.15 \times 10^{-7})(3.23 \times 10^{-4})} = \underline{9 \times 10^3 \text{ mol}^{-1} \text{ l}}$$

For Pb-HA

$$Dc = 9.8 \times 10^{-6} \cdot \left(\frac{3.8}{9.0}\right)^{3/2}$$

$$9.8 \times 10^{-6} \times 0.27 = \underline{2.6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}}$$

$$K = \frac{3.43 \times 10^{-7}}{(2.17 \times 10^{-7})(8.41 \times 10^{-6})} = \underline{1.88 \times 10^5 \text{ mol}^{-1} \text{ l}}$$

For Pb-FA

$$Dc = 9.8 \times 10^{-6} \cdot \left(\frac{3.6}{9.15}\right)^{3/2}$$

$$9.8 \times 10^{-6} \times 0.25 = \underline{2.5 \times 10^{-6}\ cm^2\ s^{-1}}$$

$$K = \frac{1.45 \times 10^{-6}}{(7.24 \times 10^{-7})(3.22 \times 10^{-4})} = \underline{6.22 \times 10^{3}\ mol^{-1}\ l}$$

From Figure 47 :- HA-Zn

$$Dc = Dnc \left(\frac{hc}{hnc}\right)^{3/2}$$

$$= 7.2 \times 10^{-6} \cdot \left(\frac{0.9}{12.5}\right)^{3/2}$$

$$= 7.2 \times 10^{-6} \times 0.02 = \underline{1.44 \times 10^{-7}\ cm^2\ s^{-1}}$$

For Zn and Cd titrated with HA and FA the voltammogram exhibits nearly the same behaviour as that of the free metal.

e) Apparatus and Instrumental Parameters

DPASV-HMDE

See Part II, Section 1. The initial and final potentials vs. SCE were as follows :-

	Zn	Cd	Pb	Cu
Initial Potential (V)	-1.2	-0.85	-0.65	-0.45
Final Potential (V)	-0.85	-0.65	-0.45	+0.15

Ultra Violet-Spectrophotometry

Apparatus

Unicam SP1700 UV Spectrophotometer

Unicam SP1805 Programme Controller

Unicam AR 25 Linear Recorder

Method

The reference cell was filled with 0.1 M NaOH. The silica cells were 1 cm^2 in dimension.

Instrumental Parameters

Unicam SP1700

Wavelength Range	200 to 390 nm

Unicam SP1805

Scan Speed	1.0 nm/s
Band Width	1.2 nm
Slit Width	0.35 nm

Unicam AR 25

Chart Speed	20 cm/s

SECTION 5 : PLANTS : METAL FRACTIONATION STUDIES

5.1 Experimental Preparation

a) Reagents

b) Sample Collection

c) Sample Treatment, Analysis and Results

d) Apparatus and Experimental Parameters

SECTION 5 : PLANTS : METAL FRACTIONATION STUDIES

5.1 Experimental preparation

a) Reagents

AnalaR potassium nitrate, calcium nitrate, ammonium dihydrogen phosphate, magnesium sulphate, ferrous sulphate, tartaric acid, boric acid, manganese chloride, cupric sulphate, zinc sulphate, molybdenum trioxide, magnesium chloride, sodium acetate, hydroxylamine, hydrogen perodixe, ammonium acetate, citric acid, sodium hydroxide, ethanol, 0.91 ammonia solution, cadmium nitrate, potassium dihydrogen phosphate, di-sodium hydrogen phosphate, ammonium oxalate, potassium hydroxide, pentan-1-ol, chloroform, ammonium chloride and ascorbic acid.

AristaR nitric acid, acetic acid, hydrochloric acid and hydrofluoric acid.

BDH general laboratory reagent grade, hexane, ethanol, acetone and ether (all distilled to the required purity). BDH chloramphenicol.

Sigma Chemical Company papain; papainase from Papaya latex crude, Type I.

b) Sample collection

Plant samples were collected from the following sites :-

Grass, Holcus lanatus from Hallen (ST 555 802) and Midger (ST 795 893) Woods.

Moss, Rhytidiadelphus squarrosus and grass, Agrostis tenuis, from Callington (SX 368 693).

Moss, Ceratodon purpureus and grass, Agrostis canina from Luckett (SX 385 736).

The samples were transferred to the laboratory in polyethylene bags.

The sampling sites are indicated in Figure 64.

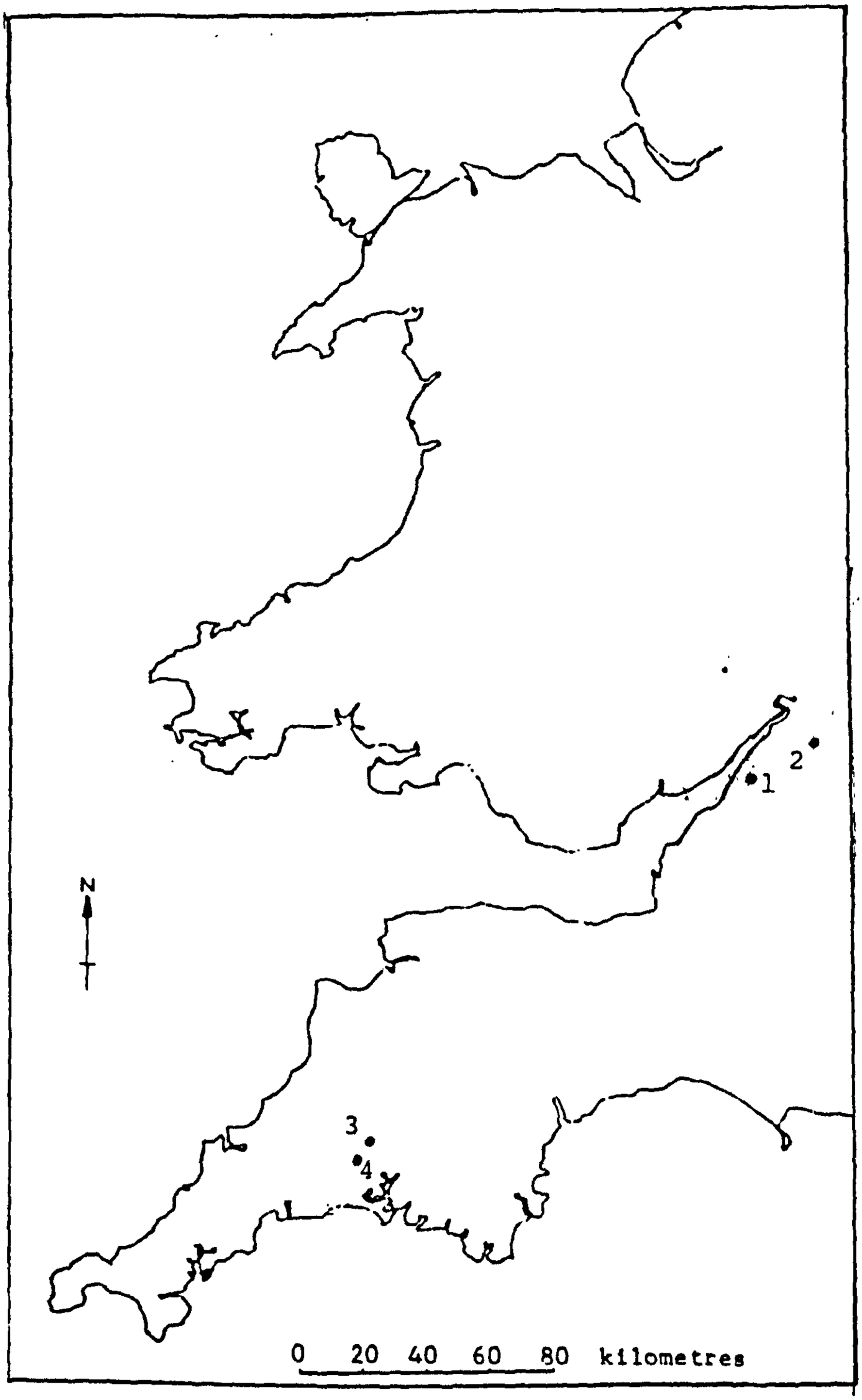


Figure 64 : Plant sampling sites

Key :- 1. Hallen
2. Midger
3. Luckett
4. Callington

c) Sample treatment, analysis and results

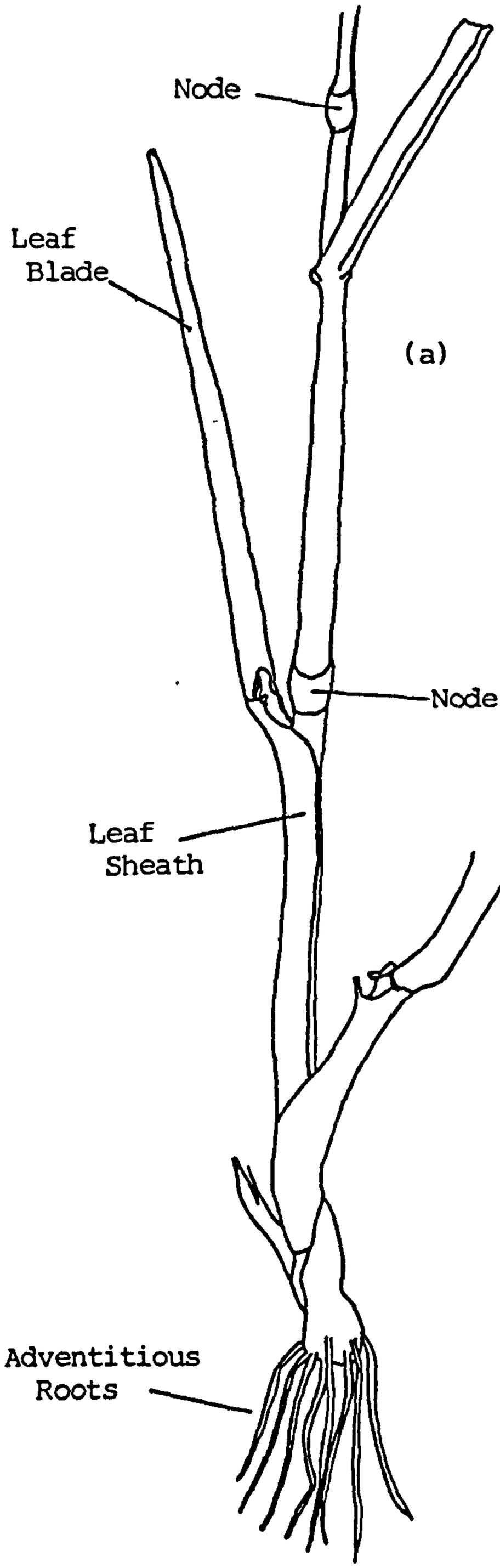
Hallen and Midger : Holcus lanatus

The samples of H. lanatus collected from Hallen and Midger woods were washed with DDW. The roots were excised at a node (the vegetative structure of grass is indicated in Figure 65). The plants were suspended in a nutrient solution as indicated in Figure 66). Hoaglands solution (289), (the composition of which is given on page 233) has been used successfully (139,140). A dilute version (the composition of which is also given on page 233) was found to give good results. Air was supplied to the new roots via a glass tube and the plants were supplied with light (18 h day 20°C, 6 h night 18°C) by two fluorescent lights.

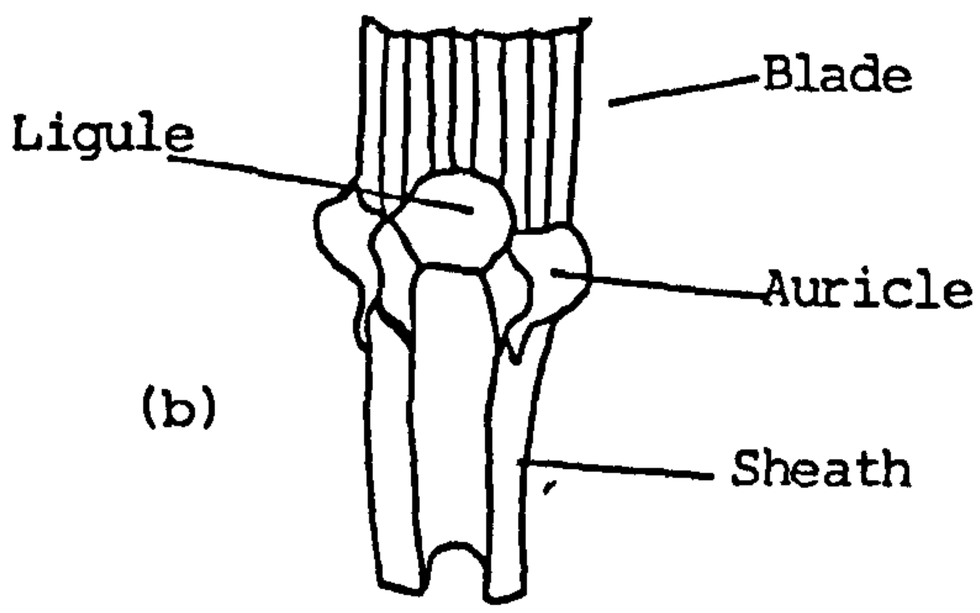
In order to prevent nutrient depletion, the nutrient solution was changed every 3 days. After 7 days, half of the plants were supplied with the nutrient solution amended with 1 µg/ml Cd^{2+} (as $CdSO_4.8H_2O$). The other half of the plants, as controls, were supplied with non-amended nutrient solution (the concentrations of Cd, Cu, Pb and Zn in the nutrient solution before addition of $CuSO_4.8H_2O$ and $ZnSO_4.8H_2O$ are given in Table 64.

After 21 days the test and control plants were removed from the nutrient solutions, their roots washed with DDW and blotted on filter paper, before being separated into roots and shoots. The roots and shoots were oven dried at 50°C, and their weights determined. The roots and shoots from both populations were subjected to the sequential extraction scheme (page 171) based on that used by Tessier et al. (241). The analysis of the extracts for Cd was carried out by FAAS (conditions, page 151) and the results are given in Table 62.

Shoots of H. lanatus collected from Hallen were (after washing with DDW) subjected to the "Quick" sequential extraction scheme outlined in Figure 40. The extracts were also analysed by FAAS. The results obtained are given in Table 63.



(a) Lower part of stem with leaves



(b) Leaf Structure

Figure 65 : Vegetative structure of grass

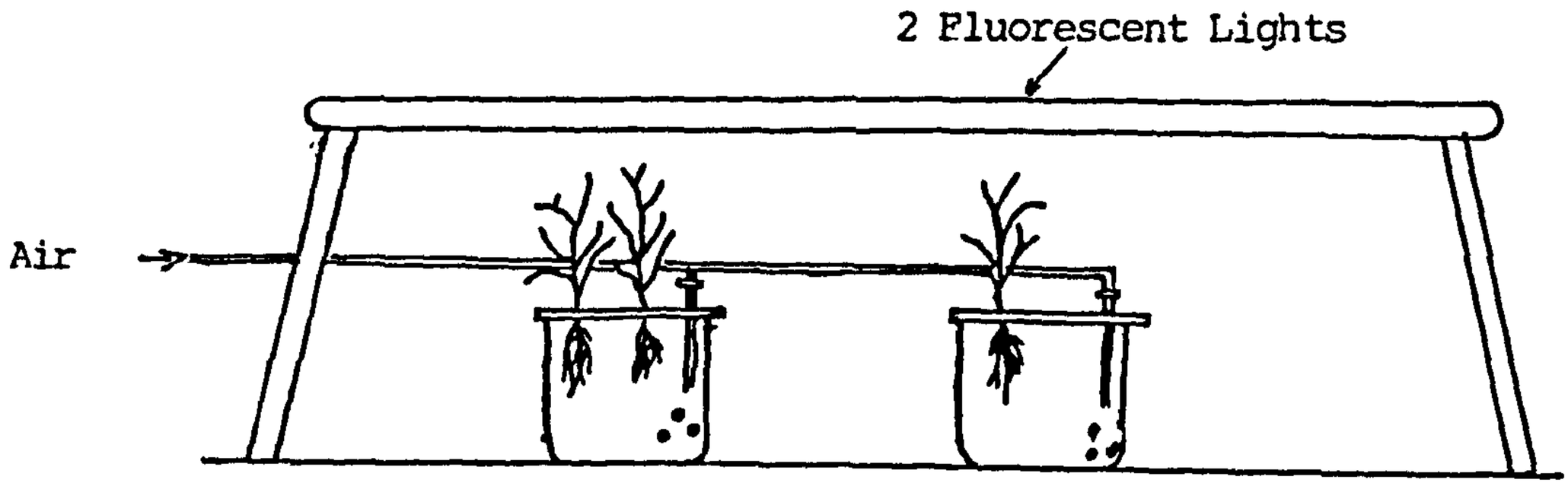


Figure 66 : Plants in nutrient culture

Nutrient solution composition

	'Randalls Recipe' g/l	'Hoagland's' g/l
KNO_3	0.253	0.656
$Ca(NO_3)_2$	0.160	0.656
$NH_4H_2PO_4$	0.0140	0.115
$MgSO_4.7H_2O$	0.048	0.490
$FeSO_4.7H_2O$ 0.5%	0.6 ml/l	0.5% 0.6 ml/l
Tartaric acid 0.4%		0.4%
H_3BO_3	0.891 mg/l	2.86 mg/l
$MnCl_2.4H_2O$	6.60 mg/l	1.81 mg/l
$CuSO_4.5H_2O$	0.026 mg/l	0.08 mg/l
$ZnSO_4.7H_2O$	0.050 mg/l	0.22 mg/l
H_2MoO_4	0.050 mg/l	0.09 mg/l

Table 62 : Fractionation of hydroponically grown H. lanatus from Hallen and Midger Woods. Analysis by FAAS

a) Roots

	Cd Concentration µg/g			
	Midger		Hallen	
Fraction	Test	Control	Test	Control
Soluble	272	1	930	1
Exch.	391	1	353	2
Carbonate	302	2	295	1
Organic	620	1	837	3
Reducible	46	2	45	1
Residual	92	2	54	3
Total	1723	9	2514	11

b) Shoots

	Cd Concentration µg/g			
	Midger		Hallen	
Fraction	Test	Control	Test	Control
Soluble	5	1	9	2
Exch.	15	1	10	1
Carbonate	5	1	3	1
Organic	23	1	22	1
Reducible	2	1	3	1
Residual	37	1	17	4
Total	87	6	64	10

Table 63 : Fractionation of Hallen H. lanatus shoots by the "Quick" fractionation scheme. Analysis by FAAS

	Concentration (µg/g)			
Fraction	Zn	Cd	Pb	Cu
Exch./Carb.	92	N.D.	11	5
Reducible	4	N.D.	5	3
Organic	N.D.	N.D.	8	N.D.
Residual	12	N.D.	16	22
Total	108	-	40	30
Acid digest (HF/HNO_3)	109	N.D.	44	31

Table 64 : Background (i.e. no Cd, Cu, Pb or Zn added) concentrations of nutrient solution. Analysis by FAAS

	Concentration (µg/ml)			
	Zn	Cd	Pb	Cu
Nutrient solution	N.D.	N.D.	0.1	0.1

Contaminated peat samples

A sample (4 g) of the Cu-peat prepared in Section 3 was fractionated using the sequential extraction scheme outlined in Figure 67. A Cu-peat sample (1 g) was also ashed according to the procedure given in Figure 68.

The fractions were obtained as follows :-

The dried, ground peat sample was refluxed with 150 ml of 80% v/v ethanol, for 20 minutes. After cooling, the mixture was filtered under vacuum through a 5 μm pore size filter, using a Buchner apparatus. The residue was refluxed with 100 ml DDW for 10 minutes, allowed to cool and then filtered through a 5 μm pore size filter. The residue from the DDW reflux was dried, acid digested with concentrated HNO_3, filtered through Whatman 541 filter paper and analysed by FAAS using the conditions given on page 151. The filtrate from the DDW reflux was divided into two volumes of approximately 50 ml each. One of these was acid digested with concentrated HNO_3, allowed to cool, neutralised to a pH of 3 with ammonia solution (0.88)) and examined by DPASV-HMDE at pH 3 (0.2 M ammonium citrate). The other portion of the DDW filtrate was analysed at pH 7 (0.2 M ammonium acetate), pH 5 (0.2 M sodium acetate) and pH 3 (0.2 M ammonium citrate) using DPASV-HMDE.

The filtrate from the ethanol reflux was rotary evaporated (Buchl. Rotavapor, 45°C, Speed ~ 0.5 rpm) to remove the alcohol. On cooling, 100 ml DDW was added in order to dissolve the residue. The solution was divided into two volumes of approximately 50 ml each. One of these was acid digested as described above and examined by DPASV-HMDE at pH 3, the other 50 ml fraction was analysed at pH 7, pH 5 and pH 3 by DPASV-HMDE.

The results obtained are given in Table 65 and a voltammogram of the ethanol reflux filtrate at pH 3 and < 5 μm is shown in Figure 69.

Figure 67 : Fractionation of Cu-Peat. Analysis by FAAS and DPASV-HMDE

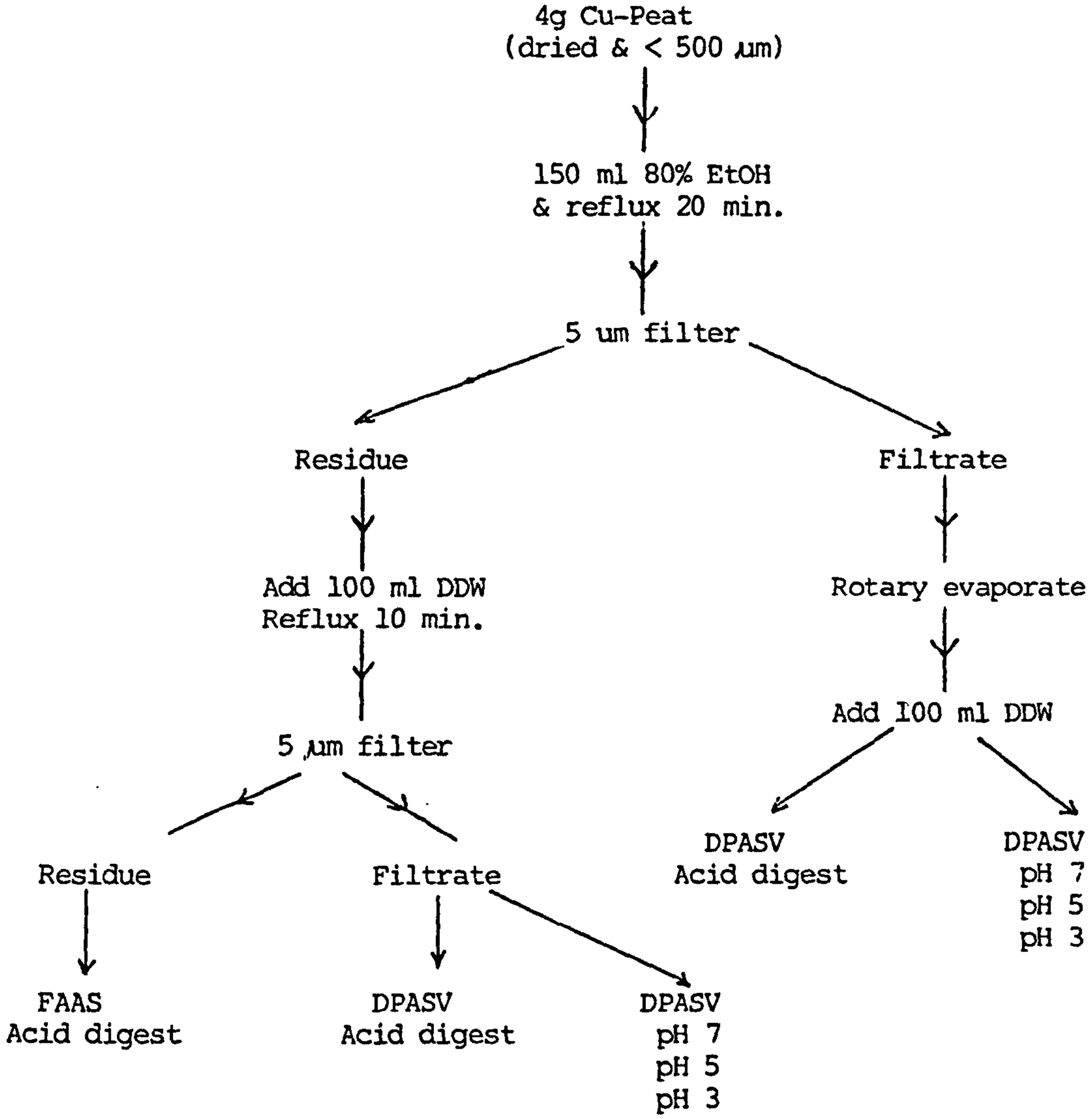


Figure 68 : Cu-peat : Ashing procedure

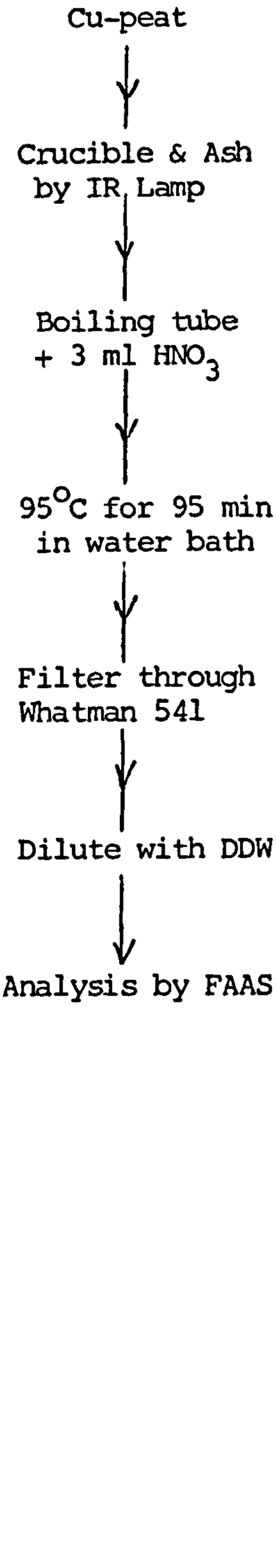
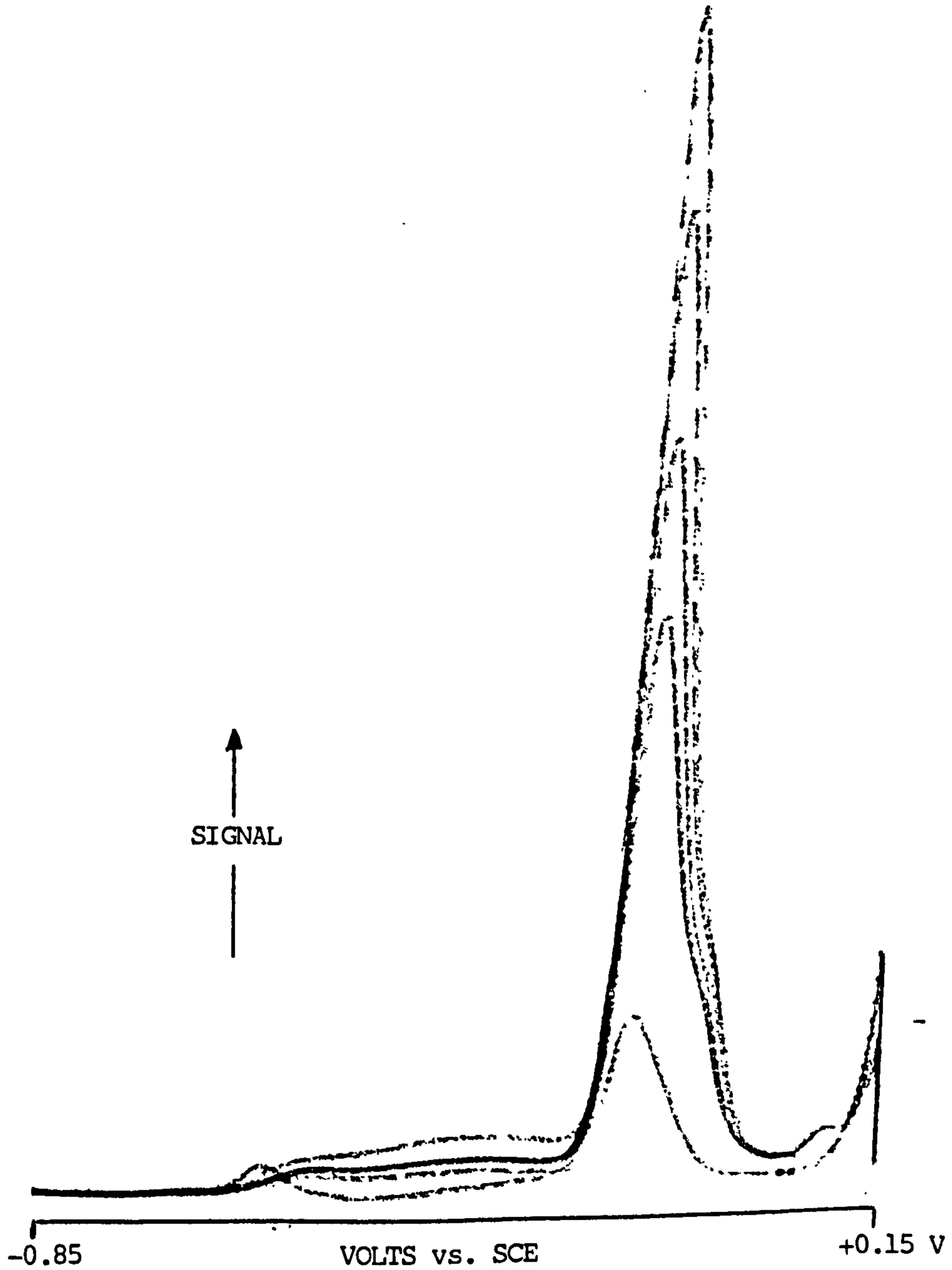


Table 65 : Fractionation of Cu-peat. Analysis by DPASV-HMDE and FAAS

	Cu Concentration (µg/g)	
Fraction	DDW REFLUXED RESIDUE > 5.0 µm	EtOH REFLUXED FILTRATE < 5.0 µm
pH 7	1	136
pH 5	1	317
pH 3	17	635
Acid digest	24	678
Residue Acid digest	1701*	-
Ash. Acid digest		2414*

* FAAS determination

Figure 69 : Differential pulse stripping curve (HMDE) of the ethanol reflux filtrate of Cu-peat at pH 3 (0.2 M ammonium citrate) and < 5 µm



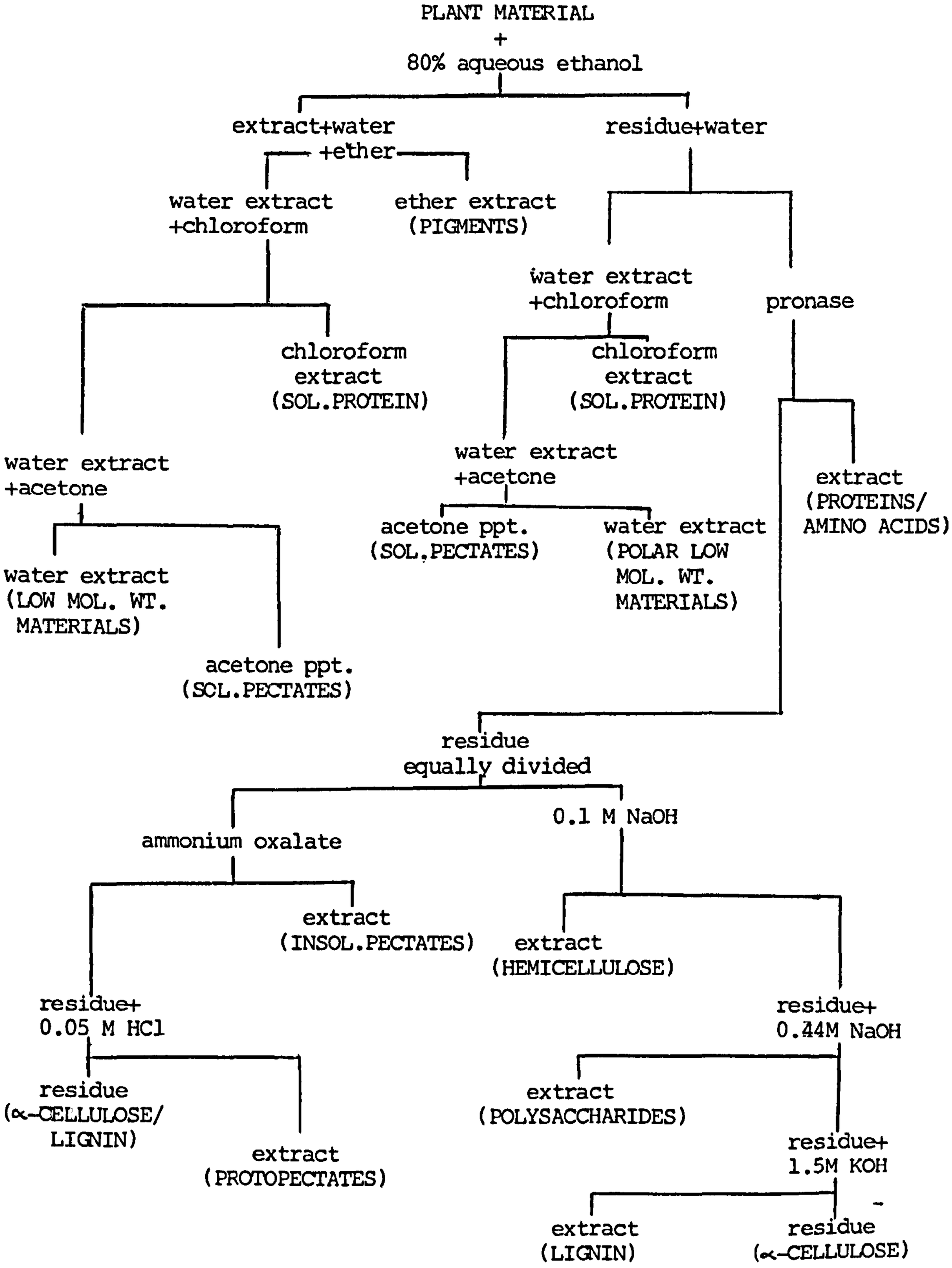
The Cu-peat (1 g) was placed in a crucible under an IR lamp, After ashing the peat and determining the ash weight, the ash was placed in a boiling tube with 3 ml concentrated HNO_3. The acid mixture was heated on a water bath at 95 $\pm$ 3°C for 95 minutes. On cooling the mixture was filtered through Whatman 541 filter paper, and made up to 50 ml with DDW, analysis was carried out by FAAS. The results are given in Table 65.

Metal containing peat samples, such as Cu-peat (freshly prepared using 0.2 M $CuSO_4.5H_2O$) and Cd-peat (freshly prepared using $Cd(NO_3)_2$) were fractionated using a modified version of the Farago et al. (126) scheme (given in full in Figure 70).

The Cu- and Cd-peat samples were fractionated as follows :-

The dried peat sample (4 g) was placed in a 250 ml glass-stoppered conical flask (Pyrex). An 80% v/v aqueous ethanol solution (150 ml) was added to the flask. The mixture was refluxed for 15 minutes and then filtered through a 5 µm filter. The filtrate (P) was stored and examined by FAAS. The residue (A) was dried at 50°C. DDW (100 ml) was added to the dried residue, and the mixture refluxed for 10 minutes, after which it was filtered through a 5 µm filter. The filtrate (C) was stored and analysed by FAAS. The residue (B) was dried and then mechanically shaken with 200 ml of phosphate buffer, pH 7.4 (prepared by mixing 0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4 in a ratio of 1:1), 10 mg of chloramphenicol and 2 g of papain for approximately 40 hours. The mixture was filtered through a 5µm filter and the filtrate (H_1) stored. The papain treatment of the residue was repeated. After filtration, the filtrate (H_2) was combined with (H_1), and analysed by FAAS. The residue was dried, and divided accurately into two equal portions (J' and J"). One half (J') of the residue was mechanically shaken with 100 ml of 2% w/v ammonium oxalate solution for 1 h, before being

Figure 70 : Scheme for extraction procedure (Farago et al. (126))



centrifuged at 3500 rpm for 30 minutes. The supernatant (M) was analysed by FAAS. The residue (N) was dissolved in 25 ml of 0.05 M hydrochloric acid.

The mixture was filtered (5 μm) and the extract (X) analysed by FAAS. The residue (Y) was acid digested before analysis by FAAS.

The other half of the dried residue (J") was dissolved in 25 ml of 0.1 M sodium hydroxide. The mixture was stirred occasionally over a period of 1 hour, and was then filtered through Whatman 541 filter paper. The filtrate (L) was stored and analysed by FAAS. The residue (K) was treated with 0.44 M sodium hydroxide (25 ml), for 1 hour, with occasional stirring. The mixture was filtered (Whatman 541) and the filtrate (W) stored and analysed by FAAS. The residue (V) was treated with boiling 1.5 M potassium hydroxide (15 ml). The mixture was evaporated (via a hot plate) to ~10 ml, and was then allowed to cool, before being filtered through Whatman 541 filter paper, and diluted to 25 ml with DDW. The filtrate (Z') was analysed by FAAS and the residue (Z) acid digested and then analysed by FAAS.

The results obtained are given in Tables 66a and 67a.

Table 66a: Fractionation of Cd-peat by the modified Farago Scheme. Analysis by FAAS

Fraction		Classes of compounds	Cd µg/g (DW)	% of total Cd
Ethanol		Pigments, sol. protein, sol. pectates, low molecular weight materials	8	0.02
DDW		Sol. protein, sol. pectates, polar low molecular weight materials	375	1.0
Papain	(H)	Proteins, amino acids	4318	11.7
Ammonium oxalate	(M)	Insoluble pectates	4347	11.8
HCl	(X)	Protopectates (a)	5774	15.6
Acid digest HCl residue	(Y)	α-cellulose, lignin	7608	20.6
0.1 M NaOH	(L)	Hemicellulose	2501	6.8
0.44 M NaOH	(W)	Polysaccharides (b)	1706	4.6
1.5 M KOH	(Z')	Lignin	2803	7.6
Acid digest KOH residue	(Z)	α-cellulose	7556	20.4
Total			36996	
Acid digest	HF/ HNO_3		36999	

(a) including polysaccharides (b) including other carbohydrates

Table 67a : Fractionation of Cu-peat by the modified Farago Scheme. Analysis by FAAS

Fraction		Classes of compounds	Cu µg/g (DW)	% of total Cu
Ethanol		Pigments, sol. protein, sol. pectates, low molecular weight materials	35	0.1
DDW		Sol. protein, sol. pectates, polar low molecular weight materials	1751	6.6
Papain	(H)	Proteins, amino acids	3402	12.8
Ammonium oxalate	(M)	Insoluble pectates	9496	35.6
HCl	(X)	Protopectates (a)	2677	10.0
Acid digest HCl residue	(Y)	∝-cellulose, lignin	829	3.1
0.1 M NaOH	(L)	Hemicellulose	5081	19.1
0.44 M NaOH	(W)	Polysaccharides (b)	251	0.9
1.5 M KOH	(Z')	Lignin	604	2.3
Acid digest KOH residue	(Z)	∝-cellulose	2511	9.4
Total			26637	
Acid digest	HF/ HNO_3		26630	

(a) including polysaccharides (b) including other carbohydrates

Cornwall samples, Hallen H. lanatus and SRM 718 Orchard leaves

The Cornwall and Hallen plant samples were washed with DDW and blotted dry with filter paper. The Hallen plants were separated into roots and shoots. The samples were dried at 50°C. The plant material was finely divided - initially this was attempted using a blender assembly (Janke-Kunkel, Irka-Werk, Ultra-Turrax blender, and Janke-Kunkel Thyristor, Regler, Irka-Werk adaptor). The samples were placed in a 100 ml round bottom glass flask, supplied with the blender (Schott, flask and Buchl glass adaptor). Re-distilled hexane (75 ml) was added to the flask. It was found that the blender did not break down the fibrous plant material and as a result became blocked. The plant material was therefore finely divided using scissors.

The moss samples (R. squarrosus and C. purpureus) were not easily separated from the soil. Thus, after drying the samples were gently ground with a pestle and mortar to separate the soil, and were then sieved through a 500 μm sieve. The moss was retained, the retained fraction was re-ground and sieved again. The moss was collected as the < 500 μm fraction.

The Cornwall and Hallen samples and the SRM 718 Orchard leaves were fractionated by the Farago et al. scheme(given on page 242). The fractions were obtained as follows :-

A sample (~ 1 g) was refluxed for 15 minutes with 50 ml of 80% v/v ethanol solution. On cooling the suspension was filtered through a 5 μm filter. The residue (A) was dried at 40°C. The 80% v/v ethanol extract (P) was taken to dryness by rotary evaporation at 40°C. The dried extract was dissolved in 35 ml DDW and extracted with ether (40 ml). The ether extract (R) was taken to dryness, wet ashed and analysed by FAAS. The water layer (Q) was shaken with pentan-1-ol (10 ml) and chloroform (25 ml) for 15 minutes. The lower portion was run off,

taken to dryness by rotary evaporation, acid digested and the metal content determined. Traces of volatile organic solvents were removed from the upper, mainly aqueous layer (T) by passing a stream of nitrogen into this layer. An equal volume of acetone was added to produce a precipitate (U). The mixture was centrifuged at 3000 rpm for 15 minutes. The supernatant (V) was decanted and both samples (U and V) were wet-ashed and analysed.

Residue (A) from the first 80% v/v ethanol treatment was refluxed with DDW (35 ml) for 10 minutes. The residue (B) was filtered off, using a 5 µm filter, and dried at 40°C. The dried extract (C) was dissolved in 15 ml DDW and the pentan-1-ol-chloroform-acetone treatment carried out as previously to give samples (D), (E), (G) and (F).

Residue (B) was shaken with 0.5 g papain, 3 to 4 mg chloramphenicol and 65 ml of phosphate buffer (pH 7.4) for 40 h. The sample was filtered off (5 µm pore size, filter) and the papain treatment repeated. The resultant extracts (H) were wet-ashed. The residue (J) was washed with ~30 ml DDW, dried and accurately divided into two parts. The first portion (J') was shaken with 2% w/v ammonium oxalate (35 ml) for 2 h. After centrifugation (3000 rpm, 15 minutes), extract (M) was analysed, and the residue (N) heated with 0.05 M hydrochloric acid to give samples (X) and (Y).

The second portion (J") was extracted successively with 0.1 M and 0.44 M sodium hydroxide, followed by 1.5 M potassium hydroxide (boiling), as described previously. The successive treatments yielded samples (L), (K), (W), (V) and (Z). The results of the analyses are given in Tables 66b to 79.

Table 66b: Fractionation of SRM 718 Orchard leaves. Analysis by FAAS

Zn Concentration µg/g (DW)

Fraction		SRM 718	% of total Zn
Pigments	(R)	N.D.	-
Sol. protein	(S)	N.D.	-
Sol. pectates	(U)	*	-
Low M(r) materials	(V)	N.D.	-
Sol. protein	(D)	N.D.	-
Sol. pectates	(F)	N.D.	-
Polar low M(r) materials	(G)	N.D.	-
Proteins/ amino acids	(H)	17.3	59.0
Insol. pectates	(M)	N.D.	-
Protopectates (a)	(X)	N.D.	-
∝-cellulose lignin	(Y)	5.9	20.1
Hemicellulose	(L)	1.8	6.1
Polysaccharides (b)	(W)	N.D.	-
Lignin	(Z')	4.3	14.7
∝-cellulose	(Z)	N.D.	-
Total		29.3	
Acid digest (HF/HNO_3)		17.8	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

Table 67b: Fractionation of SRM 718 Orchard leaves. Analysis by FAAS

Pb concentration µg/g (DW)

Fraction		SRM 718	% of total Pb
Pigments	(R)	N.D.	-
Sol. protein	(S)	N.D.	-
Sol. pectates	(U)	*	-
Low M(r) materials	(V)	N.D.	-
Sol. protein	(D)	N.D.	-
Sol. pectates	(F)	N.D.	-
Polar low M(r) materials	(G)	*	-
Proteins/ amino acids	(H)	13.0	41.7
Insol. pectates	(M)	N.D.	-
Protopectates (a)	(X)	N.D.	-
∝-cellulose lignin	(Y)	4.5	14.4
Hemicellulose	(L)	N.D.	-
Polysaccharides (b)	(W)	N.D.	-
Lignin	(Z')	N.D.	-
∝-cellulose	(Z)	13.7	43.9
Total		31.2	
Acid digest (HF/HNO_3)		42.3	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

Fractionation of SRM 718 Orchard leaves. Analysis of Cd by FAAS.

No Cd was detected in any of the fractions, but 0.13 µg/g Cd was found in the acid digest.

Table 68 : Fractionation of SRM 718 Orchard leaves. Analysis by FAAS.

Cu Concentration µg/g (DW)

Fraction		SRM 718	% of total Cu
Pigments	(R)	N.D.	-
Sol. protein	(S)	0.5	2.5
Sol. pectates	(U)	*	-
Low M(r) materials	(V)	0.9	4.5
Sol. protein	(D)	1.3	6.6
Sol. pectates	(F)	N.D.	-
Polar low M(r) materials	(G)	0.1	0.5
Proteins/ amino acids	(H)	14.4	72.7
Insol. pectates	(M)	0.1	0.5
Protopectates (a)	(X)	1.0	5.1
∝-cellulose lignin	(Y)	0.9	4.5
Hemicellulose	(L)	0.2	1.0
Polysaccharides (b)	(W)	0.4	2.0
Lignin	(Z')	N.D.	-
∝-cellulose	(Z)	N.D.	-
Total		19.8	
Acid digest (HF/HNO_3)		9.6	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

Table 69 : Fractionation of Hallen Holcus lanatus samples by the Farago Scheme. Analysis by FAAS

Fraction		Zn Concentration µg/g (DW) Shoots	% of total Zn	roots	% of total Zn
Pigments	(R)	3	0.8	11	1.4
Sol. protein	(S)	N.D.	-	22	2.8
Sol. pectates	(U)	N.D.	-	N.D.	-
Low M(r) materials	(V)	N.D.	-	15	1.9
Sol. protein	(D)	N.D.	-	N.D.	-
Sol. pectates	(F)	1	0.3	68	8.8
Polar low M(r) materials	(G)	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	18	4.7	387	50.1
Insol. pectates	(M)	N.D.	-	47	6.1
Protopectates (a)	(X)	N.D.	-	N.D.	-
∝-cellulose lignin	(Y)	52	13.7	72	9.3
Hemicellulose	(L)	5	1.3	3	0.4
Polysaccharides (b)	(W)	N.D.	-	N.D.	-
Lignin	(Z')	48	12.6	148	19.1
∝-cellulose	(Z)	253	66.6	N.D.	-
Total		380		773	
Acid digest (HF/HNO_3)		383		765	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

Table 70 : Fractionation of Hallen Holcus lanatus samples by the Farago Scheme. Analysis by FAAS

		Cd Concentration µg/g (DW)			
Fraction		Shoots	% of total Cd	rcots	% of total Cd
Pigments	(R)	1	25.0	N.D.	-
Sol. protein	(S)	N.D.	-	N.D.	-
Sol. pectates	(U)	1	25.0	N.D.	-
Low M(r) materials	(V)	N.D.	-	N.D.	-
Sol. protein	(D)	1	25.0	3	25.0
Sol. pectates	(F)	N.D.	-	N.D.	-
Polar low M(r) materials	(G)	N.D.	-	3	25.0
Proteins/ amino acids	(H)	1	25.0	5	41.7
Insol. pectates	(M)	N.D.	-	N.D.	.
Protopectates (a)	(X)	N.D.	-	N.D.	-
∝-cellulose lignin	(Y)	N.D.	-	1	8.3
Hemicellulose	(L)	N.D.	-	N.D.	-
Polysaccharides (b)	(W)	N.D.	-	N.D.	-
Lignin	(Z')	N.D.	-	N.D.	-
∝-cellulose	(Z)	N.D.	-	N.D.	-
Total		4		12	
Acid digest (HF/HNO_3)		5		28	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

Table 71 : Fractionation of Hallen Holcus lanathus samples by the Farago Scheme. Analysis by FAAS

		Pb Concentration µg/g (DW)			
Fraction		Shoots	% of total Pb	Roots	% of total Pb
Pigments	(R)	N.D.	-	N.D.	-
Sol. protein	(S)	N.D.	-	N.D.	-
Sol. pectates	(U)	N.D.	-	N.D.	-
Low M(r) materials	(V)	N.D.	-	N.D.	-
Sol. protein	(D)	N.D.	-	N.D.	-
Sol. pectates	(F)	N.D.	-	N.D.	-
Polar low M(r) materials	(G)	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	17	39.5	101	25.2
Insol. pectates	(M)	N.D.	-	34	8.5
Protopectates (a)	(X)	1	2.3	7	1.7
∝-cellulose lignin	(Y)	17	39.5	154	38.4
Hemicellulose	(L)	N.D.	-	6	1.5
Polysaccharides (b)	(W)	N.D.	-	6	1.5
Lignin	(Z')	N.D.	-	22	5.5
∝-cellulose	(Z)	8	18.6	71	17.7
Total		43		401	
Acid digest (HF/HNO_3)		45		404	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

Table 72 : Fractionation of Hallen Holcus lanatus samples by the Farago Scheme. Analysis by FAAS

Fraction		Cu Concentration µg/g (DW) Shoots	% of total Cu	Roots	% of Total Cu
Pigments	(R)	N.D.	-	N.D.	-
Sol. protein	(S)	N.D.	-	16	24.2
Sol. pectates	(U)	N.D.	-	9	13.6
Low M(r) materials	(V)	N.D.	-	N.D.	-
Sol. protein	(D)	N.D.	-	7	10.6
Sol. pectates	(F)	N.D.	-	N.D.	-
Polar low M(r) materials	(G)	N.D.	-	5	7.6
Proteins/ amino acids	(H)	34	50.7	N.D.	-
Insol. pectates	(M)	1	1.5	2	3.0
Protopectates (a)	(X)	1	1.5	5	7.6
∝-cellulose lignin	(Y)	10	14.9	10	15.2
Hemicellulose	(L)	1	1.5	3	4.5
Polysaccharides (b)	(W)	5	7.5	9	13.6
Lignin	(Z')	N.D.	-	N.D.	-
∝-cellulose	(Z)	15	23.4	N.D.	-
Total		67		66	
Acid digest (HF/HNO_3)		66		63	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

Table 73 : Fractionation of Callington plant samples by the Farago Scheme. Analysis by FAAS

Zn Concentration µg/g (DW)

Fraction		CM	% of total Zn	CG	% of total Zn
Pigments	(R)	N.D.	-	N.D.	-
Sol. protein	(S)	N.D.	-	N.D.	-
Sol. pectates	(U)	N.D.	-	N.D.	-
Low M(r) materials	(V)	N.D.	-	21	7.7
Sol. protein	(D)	N.D.	-	29	10.6
Sol. pectates	(F)	N.D.	-	N.D.	-
Polar low M(r) materials	(G)	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	3	3.5	44	16.1
Insol. pectates	(M)	14	16.5	N.D.	-
Protopectates (a)	(X)	N.D.	-	8	2.9
∝-cellulose lignin	(Y)	38	44.7	63	23.0
Hemicellulose	(L)	1	1.2	12	4.4
Polysaccharides (b)	(W)	N.D.	-	17	6.2
Lignin	(Z')	29	34.1	80	29.2
∝-cellulose	(Z)	N.D.	-	N.D.	-
Total		85		274	
Acid digest (HF/HNO_3)		90		280	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

CM = Callington moss sample. (Rhyatidiadelphus squarrosus)

CG = Callington grass sample (Agrostis tenuis)

Table 74 : Fractionation of Callington plant samples by the Farago Scheme. Analysis by FAAS

Fraction		Pb Concentration µg/g (DW) CM	% of total Pb	CG	% of total Pb
Pigments	(R)	N.D.	-	N.D.	-
Sol. protein	(S)	N.D.	-	N.D.	-
Sol. pectates	(U)	2	1.7	N.D.	-
Low M(r) materials	(V)	N.D.	-	23	6.4
Sol. protein	(D)	N.D.	-	N.D.	-
Sol. pectates	(F)	N.D.	-	N.D.	-
Polar low M(r) materials	(G)	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	22	18.6	13	3.6
Insol. pectates	(M)	2	1.7	2	0.6
Protopectates (a)	(X)	N.D.	-	N.D.	-
∝-cellulose lignin	(Y)	51	43.2	192	53.2
Hemicellulose	(L)	N.D.	-	1	0.3
Polysaccharides (b)	(W)	N.D.	-	N.D.	-
Lignin	(Z')	7	5.9	29	8.0
∝-cellulose	(Z)	34	28.8	101	28.0
Total		118		361	
Acid digest (HF/HNO_3)		124		360	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

No Cd was found in CM. In CG 1 µg/g (33.3%) Cd was found in fraction D and 2 µg/g (66.7%) in fraction H. The acid digest for CG contained 4 µg/g.

Table 75 : Fractionation of Callington plant samples by the Farago Scheme. Analysis by FAAS

Fraction		Cu Concentration µg/g (DW) CM	% of total Cu	CG	% of total Cu
Pigments	(R)	2	2.4	4	1.4
Sol. protein	(S)	N.D.	-	5	1.8
Sol. pectates	(U)	7	8.2	16	5.8
Low M(r) materials	(V)	N.D.	-	16	5.8
Sol. protein	(D)	3	3.5	4	1.4
Sol. pectates	(F)	N.D.	-	N.D.	-
Polar low M(r) materials	(G)	N.D.	-	3	1.1
Proteins/ amino acids	(H)	20	23.5	58	20.9
Insol. pectates	(M)	6	7.1	27	9.7
Protopectates (a)	(X)	7	8.2	39	14.0
∝-cellulose lignin	(Y)	18	21.2	35	12.6
Hemicellulose	(L)	1	1.2	22	7.9
Polysaccharides (b)	(W)	8	9.4	13	4.7
Lignin	(Z')	N.D.	-	N.D.	-
∝-cellulose	(Z)	13	15.3	35	12.6
Total		85		277	
Acid digest (HF/HNO_3)		80		273	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

Table 76 : Fractionation of Luckett plant samples by the Farago Scheme. Analysis by FAAS

Zn Concentration µg/g (DW)

Fraction		LM	% of total Zn	LG	% of total Zn
Pigments	(R)	N.D.	-	N.D.	-
Sol. protein	(S)	N.D.	-	N.D.	-
Sol. pectates	(U)	N.D.	-	N.D.	-
Low M(r) materials	(V)	N.D.	-	N.D.	-
Sol. protein	(D)	N.D.	-	N.D.	-
Sol. pectates	(F)	N.D.	-	N.D.	-
Polar low M(r) materials	(G)	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	N.D.	-	29	11.8
Insol. pectates	(M)	N.D.	-	N.D.	-
Protopectates (a)	(X)	1	0.5	N.D.	-
∝-cellulose lignin	(Y)	97	50.0	114	46.5
Hemicellulose	(L)	N.D.	-	N.D.	-
Polysaccharides (b)	(W)	N.D.	-	N.D.	-
Lignin	(Z')	1	0.5	2	0.8
∝-cellulose	(Z)	95	49.0	100	40.8
Total		194		245	
Acid digest (HF/HNO_3)		199		242	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

LM = Luckett moss sample (Ceratodon purpureus)

LG = Luckett grass sample (Agrostis tenuis)

Table 77 : Fractionation of Luckett plant samples by the Farago Scheme. Analysis by FAAS

Cd Concentration µg/g (DW)

Fraction		LM	% of total CD	LG	% of total Cd
Pigments	(R)	N.D.	-	N.D.	-
Sol. protein	(S)	2	11.8	N.D.	-
Sol. pectates	(U)	3	17.6	N.D.	-
Low M(r) materials	(V)	N.D.	-	N.D.	-
Sol. protein	(D)	1	5.9	N.D.	-
Sol. pectates	(F)	N.D.	-	N.D.	-
Polar low M(r) materials	(G)	6	35.3	5	100
Proteins/ amino acids	(H)	2	11.8	N.D.	-
Insol. pectates	(M)	N.D.	-	N.D.	-
Protopectates (a)	(X)	N.D.	-	N.D.	-
∝-cellulose lignin	(Y)	3	17.6	N.D.	-
Hemicellulose	(L)	N.D.	-	N.D.	-
Polysaccharides (b)	(W)	N.D.	-	N.D.	-
Lignin	(Z')	N.D.	-	N.D.	-
∝-cellulose	(Z)	N.D.	-	N.D.	-
Total		17		5	
Acid digest (HF/HNO_3)		15		5	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

Table 78 : Fractionation of Luckett plant samples by the Farago Scheme. Analysis by FAAS

Pb Concentration µg/g (DW)

Fraction		LM	% of total Pb	LG	% of total Pb
Pigments	(R)	N.D.	-	N.D.	-
Sol. protein	(S)	5	4.7	N.D.	-
Sol. pectates	(U)	N.D.	-	N.D.	-
Low M(r) materials	(V)	N.D.	-	N.D.	-
Sol. protein	(D)	N.D.	-	9	3.2
Sol. pectates	(F)	N.D.	-	N.D.	-
Polar low M(r) materials	(G)	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	4	3.7	N.D.	-
Insol. pectates	(M)	2	1.9	21	7.4
Protopectates (a)	(X)	N.D.	-	N.D.	-
∝-cellulose lignin	(Y)	68	63.6	57	20.1
Hemicellulose	(L)	N.D.	-	N.D.	-
Polysaccharides (b)	(W)	N.D.	-	26	9.2
Lignin	(Z')	28	26.2	35	12.3
∝-cellulose	(Z)	N.D.	-	136	47.9
Total		107		284	
Acid digest (HF/HNO_3)		102		285	

* = precipitate not formed

(a) including polysaccharides

(b) including other carbohydrates

Table 79 : Fractionation of Luckett plant samples by the Farago Scheme. Analysis by FAAS.

		Cu Concentration µg/g (DW)			
Fraction		LM	% of total Cu	LG	% of total Cu
Pigments	(RO	2	0.4	N.D.	-
Sol. protein	(S)	N.D.	-	N.D.	-
Sol. pectates	(U)	3	0.7	5	2.0
Low M(r) materials	(V)	3	0.7	*	-
Sol. protein	(D)	6	1.3	8	3.2
Sol. pectates	(F)	N.D.	-	N.D.	-
Polar low M(r) materials	(G)	2	0.4	3	1.2
Proteins/ amino acids	(H)	13	2.9	27	10.7
Insol. pectates	(M)	74	16.3	20	7.9
Protopectates (a)	(X)	37	8.1	27	10.7
∝-cellulose lignin	(Y)	83	18.2	66	26.1
Hemicellulose	(L)	29	6.5	18	7.1
Polysaccharides (b)	(W)	23	5.1	13	5.1
Lignin	(Z')	N.D.	-	N.D.	-
∝-cellulose	(Z)	179	39.4	66	26.1
Total		454		253	
Acid digest (HF/HNO_3)		454		244	

Commercially available metal tolerant seeds

Seeds of Merlin (Festuca rubra), Goginan (Agrostis tenuis) and Parys (A. tenuis) were planted in acid-washed agricultural silver sand. The seeds were watered with DDW. After germination the plants were watered with the nutrient solution described on pages 230 and 233. The plants were provided with a growing 18 h day ($\sim 20^{o}C$) and a 6 h night ($\sim 18^{o}C$). When they were approximately 8 to 10 cm high, some of the plants were removed from the sand. Their roots were washed in DDW to remove sand grains, prior to suspending the plants in nutrient solution (as indicated in Fig. 66). After 2 weeks, half of each population was placed in nutrient solution amended with 2.5 µg/ml of Cd^{2+} (as $CdSO_4.8H_2O$). The other half of each population was maintained in the non-amended nutrient solution. After 21 days the plants were removed from the water culture, the roots were washed with DDW and blotted dry. The plants were separated into roots and shoots and their wet weights determined. The samples were dried at $50^{o}C$, and their dry weights determined, before being finely divided. The samples were then fractionated using the Farago et al. scheme outlined in Figure 70 and described on page 242. The cadmium content of the fractions was determined by FAAS and the results obtained given in Tables 80&81 The moisture contents of the samples are given in Appendix I.

Merlin, Parys and Goginan plants grown from seed were also transferred to nutrient solutions to provide four cultures of each population (i.e. 12 cultures). One culture from each of Merlin, Goginan and Parys was treated with 2 µg Cu/ml, a second culture with 1 µg Pb/ml and a third with 2 µg Zn/ml. The fourth set of cultures was maintained with non-amended culture solution. As before, the nutrient solutions were changed every 3 days. After 21 days, the plants were removed, the roots washed and blotted and the plants separated into roots and shoots.

Table 80 : Fractionation of Goginan, Merlin and Parys plants grown in Cd amended culture solution. Analysis of Cd by FAAS

Roots

Fraction		GOGINAN Pb/Zn Tol. A. tenuis Cd µg/g	% of total Cd	MERLIN Pb/Zn Tol. F. rubra Cd µg/g	% of total Cd	PARYS Cu Tol. A. tenuis Cd µg/g	% of total Cd
Pigments	(R)	N.D.	-	N.D.	-	N.D.	-
Sol. proteins	(S)	N.D.	-	N.D.	-	3	0.2
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	236	13.3	94	6.4	285	15.0
Sol. protein	(D)	24	1.4	255	17.4	43	2.3
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	115	6.5	122	8.2	122	6.4
Proteins/ amino acids	(H)	1086	61.4	772	52.7	1000	52.7
Insol. pectates	(M)	63	3.6	53	3.6	101	5.3
Protopectates (a)	(X)	11	0.6	29	2.0	1	0.1
∝-cellulose, lignin	(Y)	128	7.2	39	2.7	131	6.9
Hemicellulose	(L)	8	0.5	20	1.4	78	4.1
Polysaccharides (b)	(W)	N.D.	-	N.D.	-	12	0.6
Lignin	(Z')	N.D.	-	N.D.	-	N.D.	-
∝-cellulose	(Z)	99	5.6	82	5.6	122	6.4
Total		1770		1466		1898	

No Cd was found in Parys plants from the non-amended culture solution, 6 µg/g Cd was found in the protein (H) fraction of Goginan roots and 8 µg/g Cd was found in the protein (H) fraction of Merlin roots.

Table 81 : Fractionation of Goginan, Merlin and Parys plants grown in Cd amended culture solution. Analysis of Cd by FAAS

Shoots

		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
Fraction		Cd µg/g	% of total Cd	Cd µg/g	% of total Cd	Cd µg/g	% of total Cd
Pigments	(R)	N.D.	-	N.D.	-	N.D.	-
Sol. proteins	(S)	N.D.	-	N.D.	-	N.D.	-
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	2	1.0	17	6.1	N.D.	-
Sol. protein	(D)	3	1.6	N.D.	-	8	3.9
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	2	1.0	9	3.2	6	2.9
Proteins/ amino acids	(H)	70	36.3	98	35.0	27	13.1
Insol. pectates	(M)	53	27.5	52	18.6	43	20.9
Protopectates (a)	(X)	6	3.1	N.D.	-	7	3.4
∝-cellulose, lignin	(Y)	16	8.3	27	9.6	41	19.9
Hemicellulose	(L)	41	21.2	55	19.6	42	20.4
Polysaccharides (b)	(W)	N.D.	-	N.D.	-	N.D.	-
Lignin	(Z')	N.D.	-	6	2.1	5	2.4
∝-cellulose	(Z)	N.D.	-	16	5.7	27	13.1
Total		193		280		206	

No Cd was found in the shoots of Goginan, Merlin, or Parys shoots of plants grown in non-amended culture solution.

Both wet and dry weights were determined. The samples were fractionated using the Farago et al. scheme described earlier (page242). The fractions were analysed by FAAS for Cu, Pb and Zn contents (all fractions were analysed for Cu and Zn). The results are given in Tables 82 to 90.

The moisture contents of the samples are given in Appendix I.

Analysis of the extracts obtained from the Cd treated samples was attempted using DPASV-RDE, but interference prevented quantitative results (Figure 71a).

Figure 71a: Differential pulse stripping curve (RDE) of Merlin (Cd) roots (fraction S)
(1 M ammonium chloride, 0.1 M citric acid & 0.025 M ascorbic acid) at pH 2.5

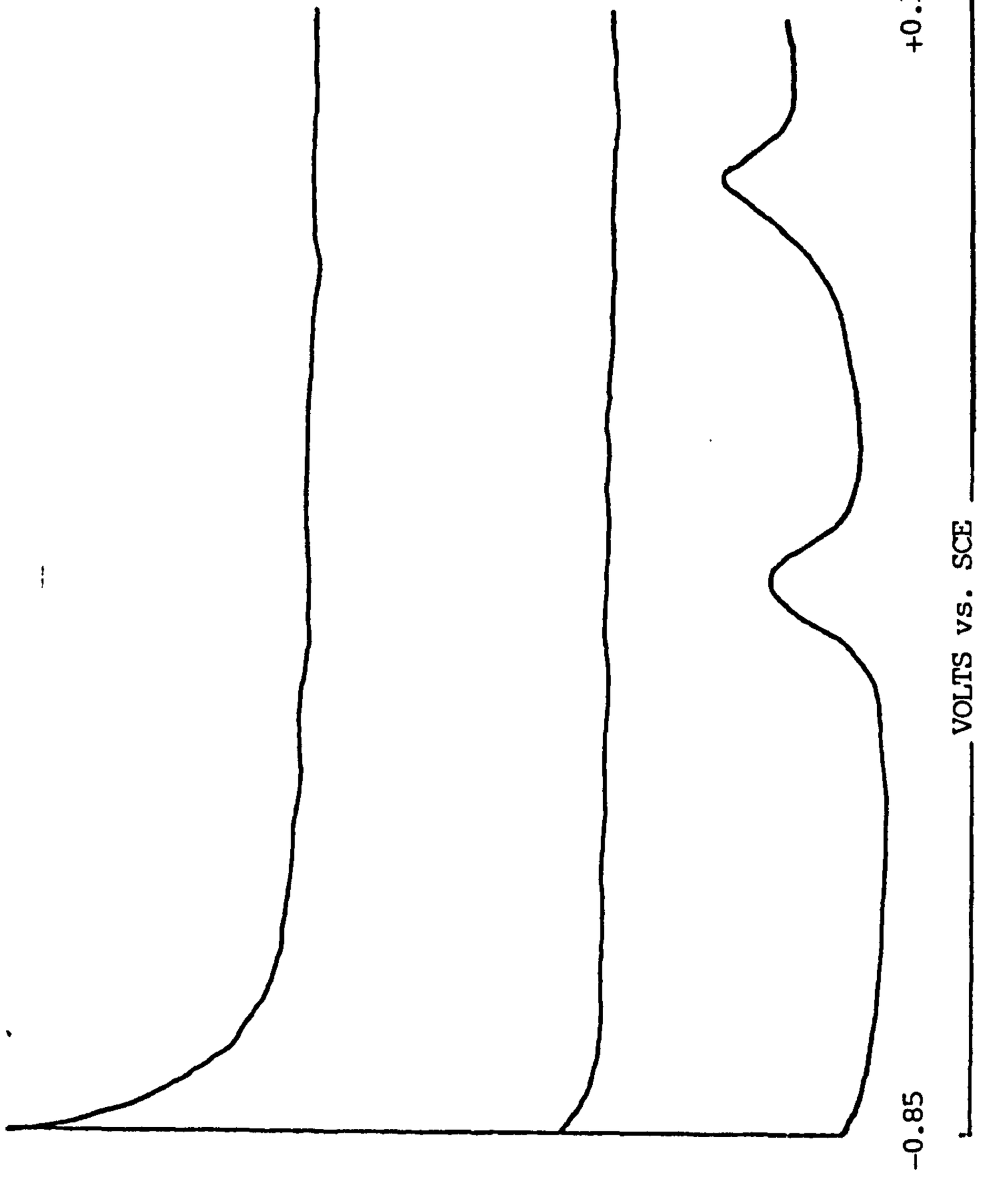


Table 82 : Fractionation of Goginan, Merlin and Parys plants grown in Pb amended culture solution. Analysis of Pb by FAAS

a) Roots

Fraction		GOGINAN Pb/Zn Tol. A. tenuis Pb µg/g	% of total Pb	MERLIN Pb/Zn Tol. F. rubra Pb µg/g	% of total Pb	PARYS Cu Tol. A. tenuis Pb µg/g	% of total Pb
Pigments	(R)	N.D.	-	N.D.	-	N.D.	-
Sol. proteins	(S)	N.D.	-	N.D.	-	N.D.	-
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	N.D.	-	N.D.	-	N.D.	-
Sol. protein	(D)	N.D.	-	33	0.9	3	0.1
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	N.D.	-	33	0.9	38	1.5
Proteins/ amino acids	(H)	258	9.3	778	20.8	1238	48.2
Insol. pectates	(M)	46	1.7	206	5.5	N.D.	-
Protopectates (a)	(X)	3	0.1	n.d.	-	29	1.1
∝-cellulose, lignin	(Y)	1189	42.8	1146	30.6	506	19.7
Hemicellulose	(L)	313	11.3	366	9.8	318	12.4
Polysaccharides (b)	(W)	176	6.3	185	4.9	N.D.	-
Lignin	(Z')	461	16.6	632	16.9	292	11.4
∝-cellulose	(Z)	331	11.9	368	9.8	144	5.6
Total		2777		3747		2568	

4 µg/g Pb was found in the hemicellulose (L) fraction of Goginan roots of plants from non-amended culture solution, 5 µg/g Pb was found in the protein (H) fraction of Merlin roots and 5 µg/g Pb was found in the polar low molecular weight (G) fraction of Parys roots.

Table 82 : Fractionation of Goginan, Merlin and Parys plants grown in Pb amended culture solution. Analysis of Pb by FAAS

b) Shoots

Fraction		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
		Pb µg/g	% of total Pb	Pb µg/g	% of total Pb	Pb µg/g	% of total Pb
Pigments	(R)	N.D.	-	N.D.	-	N.D.	-
Sol. proteins	(S)	N.D.	-	N.D.	-	N.D.	-
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	N.D.	-	N.D.	-	N.D.	-
Sol. protein	(D)	N.D.	-	4	0.7	N.D.	-
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	N.D.	-	3	0.5	N.D.	-
Proteins/ amino acids	(H)	N.D.	-	35	5.9	39	1.8
Insol. pectates	(M)	N.D.	-	43	7.2	621	29.3
Protopectates (a)	(X)	N.D.	-	19	3.2	75	3.5
∝-cellulose, lignin	(Y)	145	26.0	155	26.0	422	19.9
Hemicellulose	(L)	108	19.4	124	20.8	173	8.2
Polysaccharides (b)	(W)	83	14.9	N.D.	-	232	10.9
Lignin	(Z')	139	24.9	130	21.8	238	11.2
∝-cellulose	(Z)	83	14.9	84	14.1	321	15.1
Total		558		597		2121	

No Pb was detected in Parys shoots of plants from the non-amended culture solution, 5 µg/g Pb was found in the protein (H) fraction of Goginan shoots and 5 µg/g Pb was found in the polar low molecular weight (G) fraction of the Merlin shoots.

Table 83 : Fractionation of Goginan, Merlin and Parys plants grown in Pb amended culture solution. Analysis of Zn by FAAS

a) Roots

Fraction		GOGINAN Pb/Zn Tol. A. tenuis Zn µg/g	% of total Zn	MERLIN Pb/Zn Tol. F. rubra Zn µg/g	% of total Zn	PARYS Cu Tol. A. tenuis Zn µg/g	% of total Zn
Pigments	(R)	N.D.	-	183	18.0	140	8.0
Sol. proteins	(S)	50	4.7	339	33.3	300	17.0
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	N.D.	-	N.D.	-	N.D.	-
Sol. protein	(D)	106	9.9	133	13.1	175	9.9
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	683	64.0	87	8.6	770	43.7
Proteins/ amino acids	(H)	N.D.	-	N.D.	-	N.D.	-
Insol. pectates	(M)	53	5.0	N.D.	-	N.D.	-
Protopectates (a)	(X)	43	4.0	116	11.4	43	2.4
∝-cellulose, lignin	(Y)	73	6.8	112	11.0	78	4.4
Hemicellulose	(L)	N.D.	-	N.D.	-	208	11.8
Polysaccharides (b)	(W)	N.D.	-	N.D.	-	N.D.	-
Lignin	(Z')	60	5.6	47	4.6	47	2.7
∝-cellulose	(Z)	N.D.	-	N.D.	-	N.D.	-
Total		1068		1017		1761	

Table 83 : Fractionation of Goginan, Merlin and Parys plants grown in Pb amended culture solution. Analysis of Zn by FAAS

b) Shoots

		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
Fraction		Zn µg/g	% of total Zn	Zn µg/g	% of total Zn	Zn µg/g	% of total Zn
Pigments	(R)	53	13.5	N.D.	-	N.D.	-
Sol. proteins	(S)	48	12.2	43	7.7	42	4.6
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	N.D.	-	N.D.	-	N.D.	-
Sol. protein	(D)	52	13.2	190	34.1	290	31.4
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	N.D.	-	20	3.6	82	8.9
Proteins/ amino acids	(H)	N.D.	-	N.D.	-	231	25.0
Insol. pectates	(M)	70	17.8	35	6.3	120	13.0
Protopectates (a)	(X)	57	14.5	26	4.7	N.D.	-
∝-cellulose, lignin	(Y)	43	10.9	63	11.3	N.D.	-
Hemicellulose	(L)	28	7.1	38	6.8	48	5.2
Polysaccharides (b)	(W)	N.D.	-	24	4.3	N.D.	-
Lignin	(Z')	42	10.7	56	10.1	61	6.6
∝-cellulose	(Z)	N.D.	-	62	11.1	49	5.2
Total		393		557		923	

Table 84 : Fractionation of Goginan, Merlin and Parys plants grown in Pb amended culture solution. Analysis of Cu by FAAS

a) Roots

		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
Fraction		Cu µg/g	% of total Cu	Cu µg/g	% of total Cu	Cu µg/g	% of total Cu
Pigments	(R)	N.D.	-	45	20.3	9	1.9
Sol. proteins	(S)	N.D.	-	21	9.5	125	26.6
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	20	100.0	76	34.2	157	33.4
Sol. protein	(D)	N.D.	-	N.D.	-	N.D.	-
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	N.D.	-	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	N.D.	-	N.D.	-	125	26.6
Insol. pectates	(M)	N.D.	-	N.D.	-	N.D.	-
Protopectates (a)	(X)	N.D.	-	N.D.	-	N.D.	-
∝-cellulose, lignin	(Y)	N.D.	-	N.D.	-	45	9.6
Hemicellulose	(L)	N.D.	-	44	19.8	N.D.	-
Polysaccharides (b)	(W)	N.D.	-	N.D.	-	9	1.9
Lignin	(Z')	N.D.	-	N.D.	-	N.D.	-
∝-cellulose	(Z)	N.D.	-	36	16.2	N.D.	-
Total		20		222		470	

Table 84 : Fractionation of Goginan, Merlin and Parys plants grown in Pb amended culture solution. Analysis of Cu by FAAS

b) Shoots

		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
Fraction		Cu µg/g	% of total Cu	Cu µg/g	% of total Cu	Cu µg/g	% of total Cu
Pigments	(R)	N.D.	-	N.D.	-	N.D.	-
Sol. proteins	(S)	6	15.8	2	3.6	59	47.6
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	18	47.4	6	10.9	38	30.6
Sol. protein	(D)	N.D.	-	N.D.	-	N.D.	-
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	N.D.	-	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	N.D.	-	N.D.	-	19	15.3
Insol. pectates	(M)	N.D.	-	N.D.	-	N.D.	-
Protopectates (a)	(X)	N.D.	-	16	29.1	N.D.	-
∝-cellulose, lignin	(Y)	N.D.	-	27	49.1	8	6.5
Hemicellulose	(L)	N.D.	-	4	7.3	N.D.	-
Polysaccharides (b)	(W)	14	36.8	N.D.	-	N.D.	-
Lignin	(Z')	N.D.	-	N.D.	-	N.D.	-
∝-cellulose	(Z)	N.D.	-	N.D.	-	N.D.	-
Total		38		55		124	

Table 85 : Fractionation of Goginan, Merlin and Parys plants grown in non-amended culture solution. Analysis of Zn by FAAS

a) Roots

		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
Fraction		Zn µg/g	% of total Zn	Zn µg/g	% of total Zn	Zn µg/g	% of total Zn
Pigments	(R)	N.D.	-	N.D.	-	N.D.	-
Sol. proteins	(S)	636	68.7	53	17.2	583	73.8
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	N.D.	-	N.D.	-	N.D.	-
Sol. protein	(D)	N.D.	-	N.D.	-	71	9.0
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	N.D.	-	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	N.D.	-	N.D.	-	N.D.	-
Insol. pectates	(M)	132	14.3	63	20.4	17	2.2
Protopectates (a)	(X)	N.D.	-	86	27.8	N.D.	-
∝-cellulose, lignin	(Y)	77	8.3	23	7.4	47	5.9
Hemicellulose	(L)	74	8.0	N.D.	-	17	2.2
Polysaccharides (b)	(W)	N.D.	-	N.D.	-	N.D.	-
Lignin	(Z')	7	0.8	13	4.2	41	5.2
∝-cellulose	(Z)	N.D.	-	71	23.0	14	1.8
Total		926		309		790	

Table 85 : Fractionation of Goginan, Merlin and Parys plants grown in non-amended culture solution. Analysis of Zn by FAAS

b) Shoots

Fraction		GOGINAN Pb/Zn Tol. A. tenuis Zn µg/g	% of total Zn	MERLIN Pb/Zn Tol. F. rubra Zn µg/g	% of total Zn	PARYS Cu Tol. A. tenuis Zn µg/g	% of total Zn
Pigments	(R)	N.D.	-	N.D.	-	N.D.	-
Sol. proteins	(S)	N.D.	-	10	3.3	16	6.6
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	478	72.8	N.D.	-	N.D.	-
Sol. protein	(D)	15	2.3	6	2.0	1	0.4
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	2	0.3	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	N.D.	-	N.D.	-	N.D.	-
Insol. pectates	(M)	44	6.7	54	18.1	43	17.7
Protopectates (a)	(X)	N.D.	-	7	2.3	N.D.	-
∝-cellulose, lignin	(Y)	10	1.5	37	12.4	84	34.6
Hemicellulose	(L)	38	5.8	7	2.3	20	8.2
Polysaccharides (b)	(W)	N.D.	-	45	15.1	N.D.	-
Lignin	(Z')	41	6.2	75	25.1	70	28.8
∝-cellulose	(Z)	29	4.4	58	19.4	9	3.7
Total		657		299		243	

Table 86 : Fractionation of Goginan, Merlin and Parys plants grown in Zn amended culture solution. Analysis of Zn by FAAS

a) Roots

Fraction		GOGINAN Pb/Zn Tol. A. tenuis Zn µg/g	% of total Zn	MERLIN Pb/Zn Tol. F. rubra Zn µg/g	% of total Zn	PARYS Cu Tol. A. tenuis Zn µg/g	% of total Zn
Pigments	(R)	112	5.3	583	38.5	272	10.8
Sol. proteins	(S)	N.D.	-	N.D.	-	750	29.7
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	104	4.9	143	9.4	N.D.	-
Sol. protein	(D)	6	0.3	30	2.0	285	11.3
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	678	32.2	318	21.0	788	31.2
Proteins/ amino acids	(H)	N.D.	-	N.D.	-	N.D.	-
Insol. pectates	(M)	45	2.1	69	4.6	29	1.1
Protopectates (a)	(X)	41	1.9	14	0.9	105	4.2
∝-cellulose, lignin	(Y)	156	7.4	175	11.6	55	2.2
Hemicellulose	(L)	697	33.1	N.D.	-	N.D.	-
Polysaccharides (b)	(W)	53	2.5	N.D.	-	7	0.3
Lignin	(Z')	48	2.3	183	12.1	62	2.5
∝-cellulose	(Z)	168	8.0	N.D.	-	169	6.7
Total		2108		1515		2522	

Table 86 : Fractionation of Goginan, Merlin and Parys plants grown in Zn amended culture solution. Analysis of Zn by FAAS

b) Shoots

		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
Fraction		Zn µg/g	% of total Zn	Zn µg/g	% of total Zn	Zn µg/g	% of total Zn
Pigments	(R)	N.D.	-	N.D.	-	N.D.	-
Sol. proteins	(S)	22	1.7	49	6.0	58	16.6
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	N.D.	-	N.D.	-	N.D.	-
Sol. protein	(D)	52	4.1	58	7.1	18	5.2
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	95	7.4	77	9.5	88	25.2
Proteins/ amino acids	(H)	57	4.5	N.D.	-	34	9.7
Insol. pectates	(M)	101	7.9	45	5.5	19	5.4
Protopectates (a)	(X)	212	16.6	36	4.4	21	6.0
∝-cellulose, lignin	(Y)	14	1.1	92	11.3	48	13.8
Hemicellulose	(L)	17	1.3	83	10.2	N.D.	-
Polysaccharides (b)	(W)	387	30.3	N.D.	-	N.D.	-
Lignin	(Z')	319	25.0	69	8.5	63	18.1
∝-cellulose	(Z)	N.D.	-	303	37.3	N.D.	-
Total		1276		812		349	

Table 87 : Fractionation of Goginan, Merlin and Parys plants grown in Zn amended culture solution. Analysis of Cu by FAAS

a) Roots

Fraction		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
		Cu µg/g	% of total Cu	Cu µg/g	% of total Cu	Cu µg/g	% of total Cu
Pigments	(R)	N.D.	-	N.D.	-	N.D.	-
Sol. proteins	(S)	108	40.4	86	62.3	N.D.	-
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	111	41.6	40	29.0	40	29.6
Sol. protein	(D)	N.D.	-	N.D.	-	N.D.	-
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	N.D.	-	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	N.D.	-	N.D.	-	N.D.	-
Insol. pectates	(M)	N.D.	-	N.D.	-	5	3.7
Protopectates (a)	(X)	27	10.1	4	2.9	N.D.	-
∝-cellulose, lignin	(Y)	16	6.0	8	5.8	36	26.7
Hemicellulose	(L)	N.D.	-	N.D.	-	N.D.	-
Polysaccharides (b)	(W)	5	1.9	N.D.	-	N.D.	-
Lignin	(Z')	N.D.	-	N.D.	-	N.D.	-
∝-cellulose	(Z)	N.D.	-	N.D.	-	54	40.0
Total		267		138		135	

Table 87 : Fractionation of Goginan, Merlin and Parys plants grown in Zn amended culture solution. Analysis of Cu by FAAS

b) Shoots

		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
Fraction		Cu µg/g	% of total Cu	Cu µg/g	% of total Cu	Cu µg/g	% of total Cu
Pigments	(R)	N.D.	-	N.D.	-	N.D.	-
Sol. proteins	(S)	2	33.3	11	23.4	3	25.0
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	4	66.7	18	38.3	5	41.7
Sol. protein	(D)	N.D.	-	N.D.	-	N.D.	-
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	N.D.	-	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	N.D.	-	N.D.	-	N.D.	-
Insol. pectates	(M)	N.D.	-	N.D.	-	4	33.3
Protopectates (a)	(X)	N.D.	-	N.D.	-	N.D.	-
∝-cellulose, lignin	(Y)	N.D.	-	18	38.3	N.D.	-
Hemicellulose	(L)	N.D.	-	N.D.	-	N.D.	-
Polysaccharides (b)	(W)	N.D.	-	N.D.	-	N.D.	-
Lignin	(Z')	N.D.	-	N.D.	-	N.D.	-
∝-cellulose	(Z)	N.D.	-	N.D.	-	N.D.	-
Total		6		47		12	

Table 88 : Fractionation of Goginan, Merlin and Parys plants grown in non-amended culture solution. Analysis of Cu by FAAS

a) Roots

Fraction		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
		Cu µg/g	% of total Cu	Cu µg/g	% of total Cu	Cu µg/g	% of total Cu
Pigments	(R)	N.D.	-	N.D.	-	N.D.	-
Sol. proteins	(S)	N.D.	-	8	10.3	7	100.0
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	N.D.	-	1	1.3	N.D.	-
Sol. protein	(D)	N.D.	-	N.D.	-	N.D.	-
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	N.D.	-	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	N.D.	-	61	78.2	N.D.	-
Insol. pectates	(M)	44	66.7	8	10.3	N.D.	-
Protopectates (a)	(X)	N.D.	-	N.D.	-	N.D.	-
∝-cellulose, lignin	(Y)	11	16.7	N.D.	-	N.D.	-
Hemicellulose	(L)	11	16.7	N.D.	-	N.D.	-
Polysaccharides (b)	(W)	N.D.	-	N.D.	-	N.D.	-
Lignin	(Z')	N.D.	-	N.D.	-	N.D.	-
∝-cellulose	(Z)	N.D.	-	N.D.	-	N.D.	-
Total		66		78		7	

Table 88 : Fractionation of Goginan, Merlin and Parys plants grown in non-amended culture solution. Analysis of Cu by FAAS

b) Shoots

		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
Fraction		Cu µg/g	% of total Cu	Cu µg/g	% of total Cu	Cu µg/g	% of total Cu
Pigments	(R)	N.D.	-	N.D.	-	N.D.	-
Sol. proteins	(S)	19	55.9	9	100.0	5	45.5
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	N.D.	-	N.D.	-	N.D.	-
Sol. protein	(D)	N.D.	-	N.D.	-	N.D.	-
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	N.D.	-	N.D.	-	N.D.	-
Proteins/ amino acids	(H)	15	44.1	N.D.	-	N.D.	-
Insol. pectates	(M)	N.D.	-	N.D.	-	N.D.	-
Protopectates (a)	(X)	N.D.	-	N.D.	-	N.D.	-
∝-cellulose, lignin	(Y)	N.D.	-	N.D.	-	6	54.5
Hemicellulose	(L)	N.D.	-	N.D.	-	N.D.	-
Polysaccharides (b)	(W)	N.D.	-	N.D.	-	N.D.	-
Lignin	(Z')	N.D.	-	N.D.	-	N.D.	-
∝-cellulose	(Z)	N.D.	-	N.D.	-	N.D.	-
Total		34		9		11	

Table 89 : Fractionation of Goginan, Merlin and Parys plants grown in Cu amended solution. Analysis of Cu by FAAS

a) Roots

Fraction		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
		Cu µg/g	% of total Cu	Cu µg/g	% of total Cu	Cu µg/g	% of total Cu
Pigments	(R)	28	3.1	54	6.2	63	4.3
Sol. proteins	(S)	2	0.2	12	1.4	27	1.8
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	13	1.5	197	22.7	141	9.7
Sol. protein	(D)	N.D.	-	N.D.	-	N.D.	-
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	190	21.3	210	24.2	N.D.	-
Proteins/ amino acids	(H)	136	15.2	307	35.4	1224	83.8
Insol. pectates	(M)	127	14.2	31	3.6	N.D.	-
Protopectates (a)	(X)	N.D.	-	28	3.2	3	0.2
∝-cellulose, lignin	(Y)	N.D.	-	15	1.7	3	0.2
Hemicellulose	(L)	237	26.5	14	1.6	N.D.	-
Polysaccharides (b)	(W)	57	6.4	N.D.	-	N.D.	-
Lignin	(Z')	103	11.5	N.D.	-	N.D.	-
∝-cellulose	(Z)	N.D.	-	N.D.	-	N.D.	-
Total		893		868		1461	

Table 89 : Fractionation of Goginan, Merlin and Parys plants grown in Cu amended culture solution. Analysis of Cu by FAAS

b) Shoots

		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
Fraction		Cu µg/g	% of total Cu	Cu µg/g	% of total Cu	Cu µg/g	% of total Cu
Pigments	(R)	50	13.3	21	6.5	35	8.3
Sol. proteins	(S)	14	3.7	6	1.9	1	0.2
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	25	6.6	42	13.0	43	10.1
Sol. protein	(D)	N.D.	-	N.D.	-	N.D.	-
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	N.D.	-	30	9.3	N.D.	-
Proteins/ amino acids	(H)	84	22.3	78	24.2	213	50.2
Insol. pectates	(M)	7	1.9	43	13.4	7	1.7
Protopectates (a)	(X)	10	2.7	26	8.1	17	4.0
∝-cellulose, lignin	(Y)	37	9.8	38	11.8	N.D.	-
Hemicellulose	(L)	64	17.0	N.D.	-	39	9.2
Polysaccharides (b)	(W)	36	9.5	4	1.2	69	16.3
Lignin	(Z')	N.D.	-	N.D.	-	N.D.	-
∝-cellulose	(Z)	50	13.3	34	10.6	N.D.	-
Total		377		322		424	

Table 90 : Fractionation of Goginan, Merlin and Parys plants grown in Cu amended culture solution. Analysis of Zn by FAAS

a) Roots

		GOGINAN Pb/Zn Tol. A. tenuis		MERLIN Pb/Zn Tol. F. rubra		PARYS Cu Tol. A. tenuis	
Fraction		Zn µg/g	% of total Zn	Zn µg/g	% of total Zn	Zn µg/g	% of total Zn
Pigments	(R)	N.D.	-	N.D.	-	79	5.2
Sol. proteins	(S)	N.D.	-	N.D.	-	N.D.	-
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	N.D.	-	N.D.	-	N.D.	-
Sol. protein	(D)	43	10.9	194	20.8	289	18.9
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	29	7.3	373	39.9	669	43.8
Proteins/ amino acids	(H)	N.D.	-	198	21.2	N.D.	-
Insol. pectates	(M)	45	11.4	38	4.1	16	1.0
Protopectates (a)	(X)	N.D.	-	N.D.	-	11	0.7
∝-cellulose, lignin	(Y)	21	5.3	63	6.7	69	4.5
Hemicellulose	(L)	110	27.8	27	2.9	30	2.0
Polysaccharides (b)	(W)	10	2.5	N.D.	-	89	5.8
Lignin	(Z')	137	34.7	41	4.4	89	5.8
∝-cellulose	(Z)	N.D.	-	N.D.	-	185	12.1
Total		395		934		1526	

Table 90 : Fractionation of Goginan, Merlin and Parys plants grown in Cu amended culture solution. Analysis of Zn by FAAS

b) Shoots

Fraction		GOGINAN Pb/Zn Tol. A. tenuis Zn µg/g	% of total Zn	MERLIN Pb/Zn Tol. F. rubra Zn µg/g	% of total Zn	PARYS Cu Tol. A. tenuis Zn µg/g	% of total Zn
Pigments	(R)	8	2.8	2	0.4	78	9.6
Sol. proteins	(S)	3	1.0	113	22.3	127	15.6
Sol. pectates	(U)	*	-	*	-	*	-
Low M(r) materials	(V)	N.D.	-	N.D.	-	N.D.	-
Sol. protein	(D)	N.D.	-	106	20.9	83	10.2
Sol. pectates	(F)	*	-	*	-	*	-
Polar low M(r) materials	(G)	52	17.9	2	0.4	175	21.5
Proteins/ amino acids	(H)	70	24.1	N.D.	-	N.D.	-
Insol. pectates	(M)	31	10.7	28	5.5	4	0.5
Protopectates (a)	(X)	25	8.6	34	6.7	30	3.7
∝-cellulose, lignin	(Y)	40	13.8	60	11.8	115	14.1
Hemicellulose	(L)	3	1.0	N.D.	-	N.D.	-
Polysaccharides (b)	(W)	N.D.	-	N.D.	-	N.D.	-
Lignin	(Z')	58	20.0	98	19.3	51	6.3
∝-cellulose	(Z)	N.D.	-	64	12.6	152	18.7
Total		290		507		815	

d) Apparatus and Instrumental Parameters

FAAS and DPASV-HMDE

See Part II, Section 1.

DPASV-RDE

See Part II, Section 3.

SECTION 6 : CHARACTERISATION OF PLANT METALLOTHIONEIN-LIKE PROTEIN

6.1 Experimental Preparation

a) Reagents

b) Sample Collection

c) Sample Treatment, Analysis and Results

d) Apparatus and Instrumental Parameters

SECTION 6 : CHARACTERISATION OF PLANT METALLOTHIONEIN-LIKE PROTEIN

a) Reagents

AnalaR copper nitrate, tris(hydroxymethyl) methylamine, hydrochloric acid and sodium chloride.

BDH Chemicals general purpose reagent D-iso-ascorbic acid sodium salt.

Pharmacia Fine Chemicals, Blue Dextran 2000.

Molecular weight standards :-

Standard	Source	Molecular weight
B acitracin	Sigma Chemical Co.	1450
Cytochrome C	BDH Chemicals	12 200
Ribonuclease (Bovine pancrease)	BDH Chemicals	13 700
Trypsin inhibitor (Soybean)	Sigma Chemical Co.	20 100
Pepsin (Hog stomach mucosa)	Sigma Chemical Co.	34 700

b) Sample Collection

Thlaspi alpestre was collected from Charterhouse (ST 506 561) and Silene vulgaris (=cucubalus) from Shipham (ST 441 571). The plants were maintained in their own soils until required.

The sampling sites are indicated in Figure 71b.

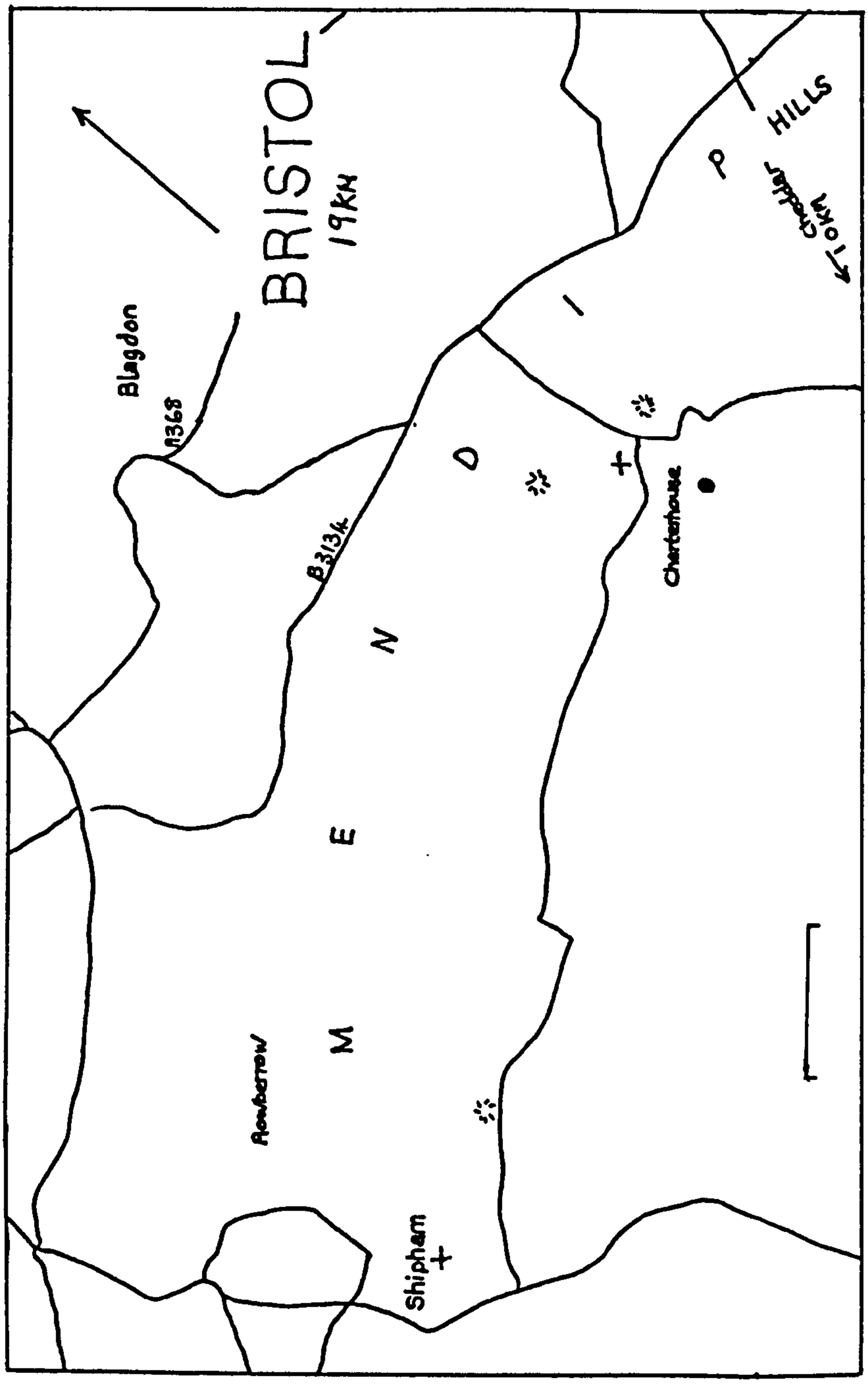


Figure 7lb: Location of plant sampling sites (for metallothionein studies)

c) Sample treatment, analysis and results

Seeds of Agrostis tenuis (Parys commercial variety, tolerant to copper) were planted in acid washed silver sand and watered with distilled water. After germination the plants were watered every 2 days with distilled water amended with 2 µg/ml Cu^{2+} (as $Cu(NO_3)_2.3H_2O$) for a period of 4 weeks. The plants were carefully removed from the growth medium and washed with SDW to remove sand particles. The plants were blotted dry with filter paper and divided into roots and shoots. The shoots and a small proportion of the roots were acid digested with concentrated HNO_3, and analysed by FAAS (using the conditions given on page 151). The remaining roots were finely divided and ground in a chilled mortar containing approximately 2 g of acid washed sand and 10 ml of a 5 mM sodium ascorbate - 50 mM Tris mixture. After grinding the brei was squeezed through 4 layers of cheesecloth (previously tested for heavy metals : Table 91). The supernatant was collected, placed in a capped plastic centrifuge tube and centrifuged in an RC-50 Superspeed refrigerated centrifuge (Du Pont Instruments/SORVALL) at 10,000 rpm for 5 minutes, using an SS-34 head (maximum operating speed = 20,000 rpm and maximum centrifugal force = 48,246 (g) RCF). The supernatant was decanted and recentrifuged at 15,000 rpm for 45 minutes. The UV absorption of this high speed supernatant was measured at 220, 230, 240, 250 and 280 nm, using a Unicam SP1700 UV Spectrophotometer, and the copper concentration determined by FAAS.

Molecular weight determination (a)

A Pharmacia Fine Chemicals SR 25/100 gel filtration column (100 cm long; i.d. 2.5 cm) was acid washed and rinsed with DDW. Sephadex G-50 fine (53 g) was placed in a 500 ml beaker. An excess of a 50 mM Tris - 50 mM NaCl mixture (pH 7.8 - by additions of concentrated HCl) was added

to the Sephadex. The slurry was stirred and then heated on a water bath for 1 hour, with occasional stirring. The excess Tris-NaCl solution and 'fines' were decanted. A further 75 ml of Tris-NaCl solution was added to the slurry and the decantation process repeated, after which the slurry was allowed to cool.

The K15/90 column was half filled with the Tris-NaCl solution and the cooled slurry poured into the column. Excess solution was allowed to run to waste. When filled with the slurry, the column was fitted to a reservoir (a 5 l beaker) containing Tris-NaCl solution. The column was washed with this solution.

The void volume (Vo) of the column was determined using 0.005 g of Dextran Blue 2000 in 0.5 ml of the Tris-NaCl solution, which was injected onto the top of the Sephadex via the injection port. The flow rate was also determined.

The column was calibrated using a series of standards of known molecular weight. Each standard (5 mg in 0.5 ml of the Tris-NaCl solution) was added to the top of the column and eluted with Tris-NaCl solution which was collected in 10 ml aliquots using a Central fraction collector. The fractions were monitored as before, using a Unicam SP1700 UV Spectrophotometer. The metal content of the standards was determined by FAAS (Table 91).

A graph of molecular weight vs. fraction volume was prepared, Figure 72.

The Parys high speed supernatant (2 ml) was added to the column, collected in 10 ml aliquots, and analysed by UV and FAAS. The molecular weight was determined from the calibration graph.

Table 91 : Concentration of Cd, Cu, Pb and Zn in reagents used

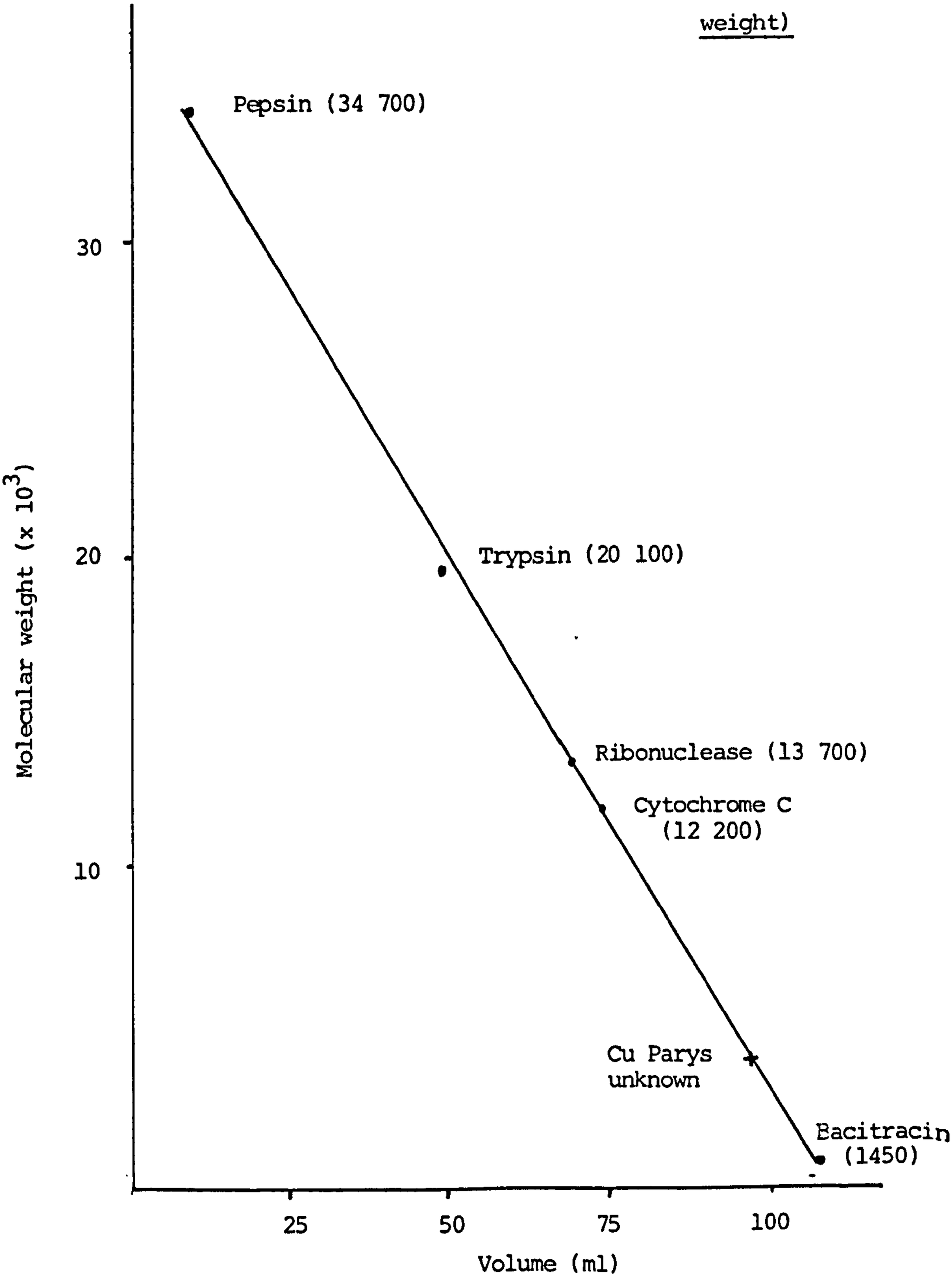
	Concentration µg/ml	
Sample	Zn	
50 mM Tris	N.D.	
50 mM Tris-50 mM NaCl	0.03	
Cytochrome C	0.70	No Cd, Pb or
Bacitracin	N.D.	Cu detected
Ribonuclease	0.11	
Trypsin inhibitor	0.03	
Pepsin	0.06	

Cheesecloth acid digest - no Zn, Cd, Pb or Cu detected

Table 92 : Void volume and flow rate of columns used

Column	Flow Rate ml/h	Void volume (ml)
Molecular weight (a)	25.0	160.0
Molecular weight (b)	5.0	50.0
Prep. gel filtration	45.5	60.0
Ion-exchange	27.0	-

Figure 72 : Molecular weight estimation of Cu-binding protein via gel filtration. Analytical gel filtration on Sephadex G-50 fine (97 x 1.2 cm) at 25 ml/h with 50 mM Tris, 50 mM NaCl at pH 7.8. Molecular weight standards are shown (molecular weight)



Preparative gel filtration

A Sephadex G-50 fine column (height 14 cm and i.d. 4.2 cm) was prepared (as for the molecular weight determination), using 50 mM Tris (pH 7.8) as eluant.

The Parys high speed supernatant (2 ml) was added to the top of the column. The eluate was collected in 10 ml volumes, which were examined by UV and FAAS as previously described. After analysis the sample containing fractions were combined.

Ion-exchange chromatography

Whatman DE52 anion exchange cellulose (5 g) was washed several times with 50 mM Tris (pH 7.8) buffer. A 1 cm (i.d.) column was filled with a bed of the anion exchange cellulose to a height of approximately 10 cm. The column was washed with 100 ml of the 50 mM Tris (pH 7.8) buffer.

The combined fractions collected from the preparative gel filtration column were added to the anion exchange column. The column was allowed to run until it was almost dry, before 50 ml of the Tris starting buffer was added. Again the column was allowed to run until nearly dry, before a linear gradient of 200 ml of NaCl from 0.2 M to 0.45 M was initiated. The column was finally washed through with starting buffer. The fractions, collected in 10 ml aliquots, were analysed as before by UV and FAAS. The sample fractions were combined and desalted by passage through a Sephadex G-25-100 (particle size 50-150 μ) column (90 cm long and 1.5 cm i.d.), eluted with DDW. The fractions were collected in 10 ml aliquots and analysed by UV and FAAS.

The procedure (i.e. sample preparation, molecular weight determination, preparative gel filtration and ion-exchange chromatography) outlined in Figure 73 was applied to the roots of Thlaspi alpestre (analysed for Zn and Cd) and the roots of Silene vulgaris (analysed for Zn and Pb - Cd was found to be 0.01 ug/ml in the high speed supernatant and was therefore not studied in the fractions collected).

Agrostis tenuis, Goginan variety (i.e. Pb/Zn tolerant) was grown as a control to the A. tenuis Parys variety (Cu tolerant). The Goginan seeds were treated in the same manner as the Parys seeds, but watered with 2 ug/ml Cu amended distilled water every 3 days for 21 days. Despite the reduction in the Cu application compared to the Parys plants, the root growth of the Goginan plants was reduced. Therefore, the whole plant was subjected to the procedure given in Figure 73.

The results are given in Figures 74 to 81 and Appendix 2.

Molecular weight determination (b)

Molecular weight determinations using the G-50 fine column indicated that the samples had lower molecular weights than the lowest standard (B acitracin 1,450 daltons). Since the molecular weight range of G-50 is from 1500 to 30,000 daltons and for G-25 is from 1000 to 5000 daltons, the molecular weight determinations were repeated on a G-25 column. The molecular weight of the Parys high speed supernatant could not be repeated due to insufficient sample being available.

Figure 73 : Characterisation of plant metal binding protein

Plant material

↓

Grind with sand and 10 ml 5 mM sodium ascorbate - 50 mM Tris

↓

Brei squeeze through cheesecloth

↓

Collect supernatant

↓

Centrifuge 10,000rpm , 5 min

↓

Collect supernatant

↓

Centrifuge 15,000 rpm, 15 min

↓

UV at 220,230,240,250 & 280 nm
FAAS analysis

↙ ↘

Molecular weight determination

SR 25/100 column
50 mM Tris - 50 mM NaCl
(pH 7.8)

a) Sephadex G-50 fine

b) Sephadex G-25 fine

Preparative gel filtration

14 x 1 cm column &
50 mM Tris (pH 7.8)

↓

Collect fractions, UV & FAAS. Combine sample fractions

↓

Ion-exchange chromatography

↓

Figure 73 continued.

Ion-exchange chromatography

1 x 10 cm column
DE52 anion exchange cellulose. Elute with

i) 50 mM Tris (pH 7.8) 50 ml

ii) 200 ml linear NaCl gradient

iii) 50 mM Tris (pH 7.8)

↓

Collect fractions, UV & FAAS. Combine sample fractions

↓

Desalt fractions
K 15/90 column
DDW as eluent

↓

Collect fractions, UV & FAAS

Figure 74 : Preparative gel filtration of a high speed supernatant from Cu treated Agrostis tenuis (Parys) roots

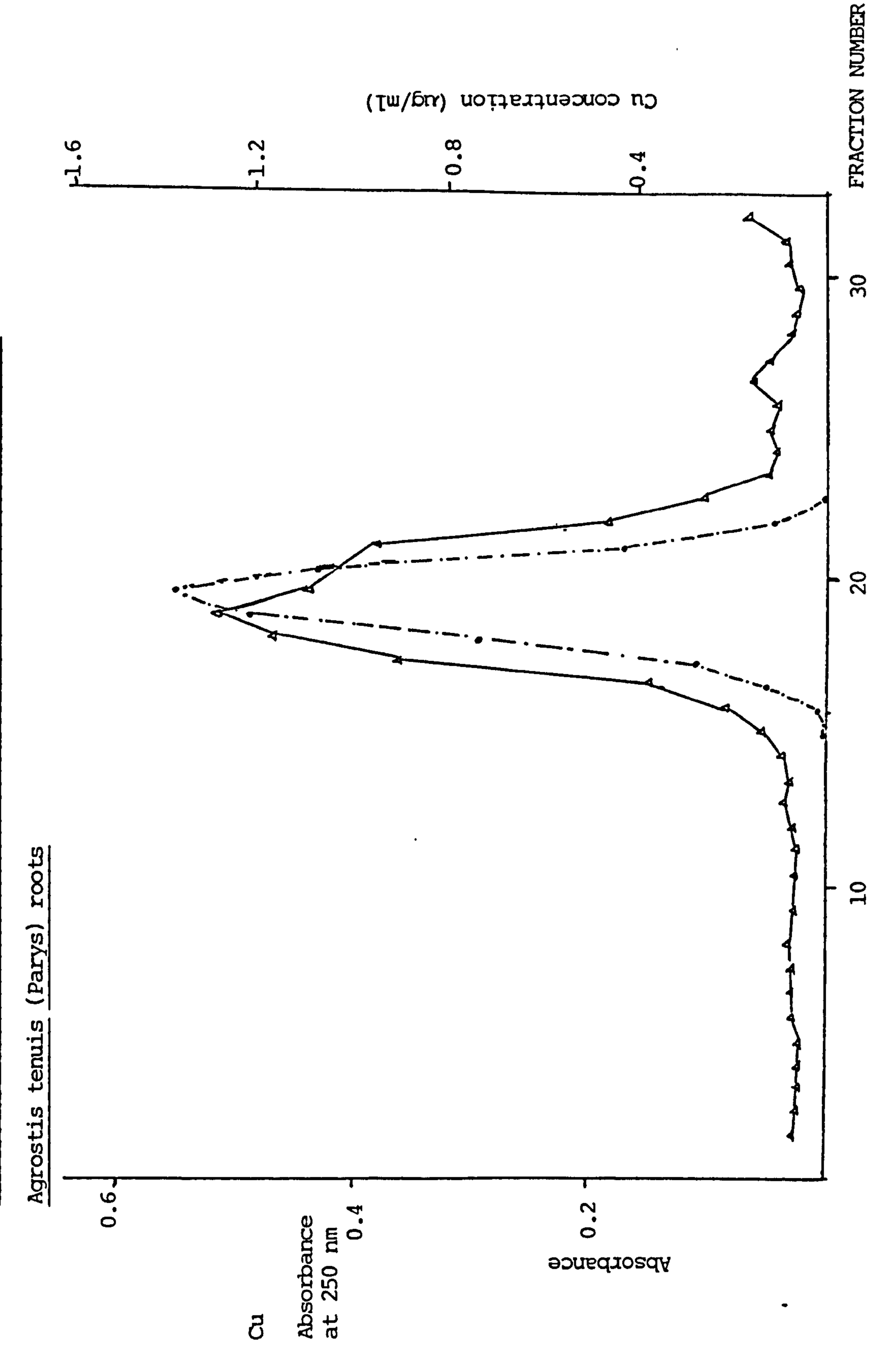


Figure 75 : DEAE-Cellulose chromatography of the Cu containing fraction of A. tenuis (Parys) roots from a Sephadex G-50 column

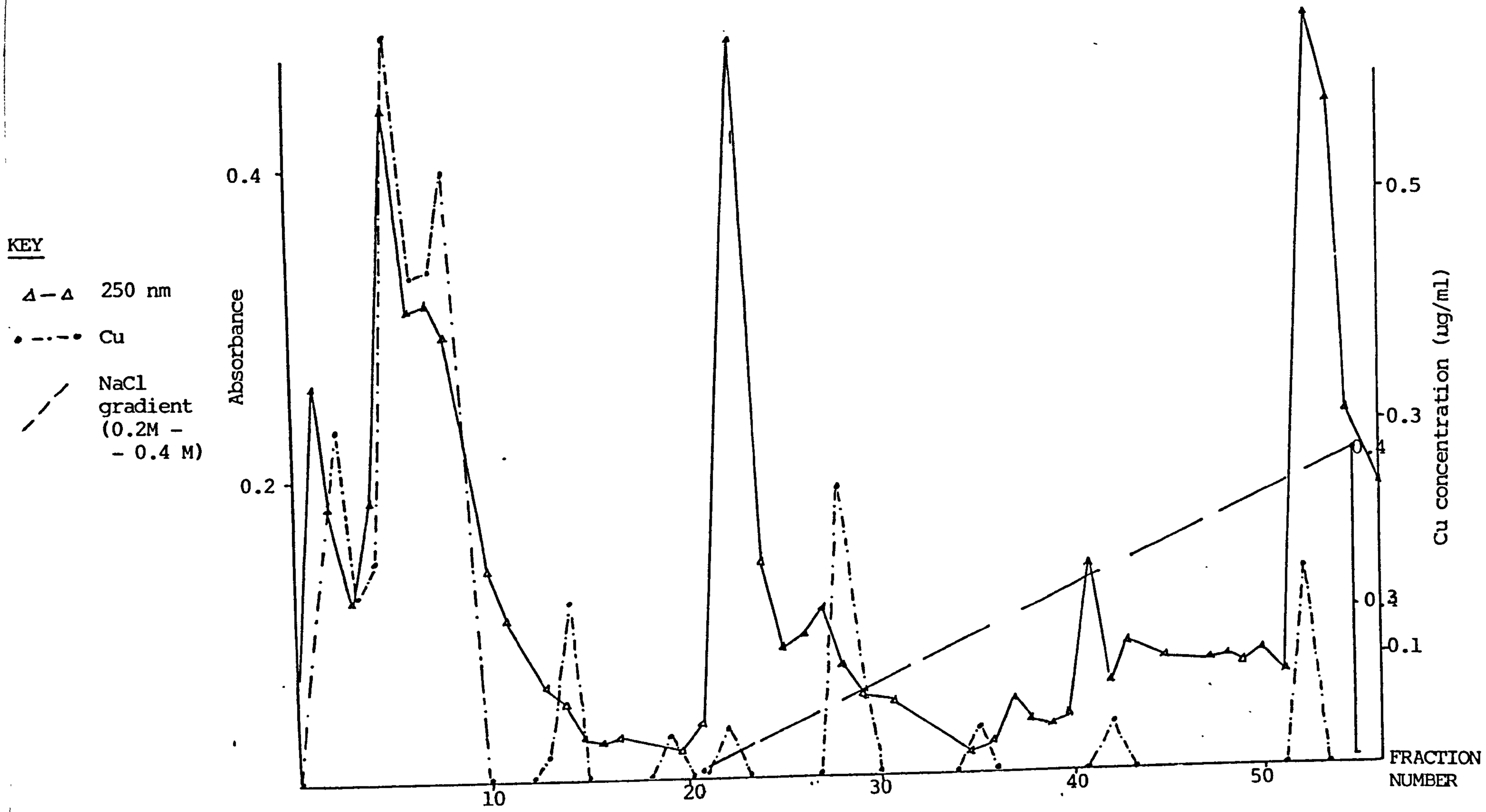


Figure 76 : Preparative gel filtration of a high speed supernatant from Cu treated Agrostis tenuis (Goginan)

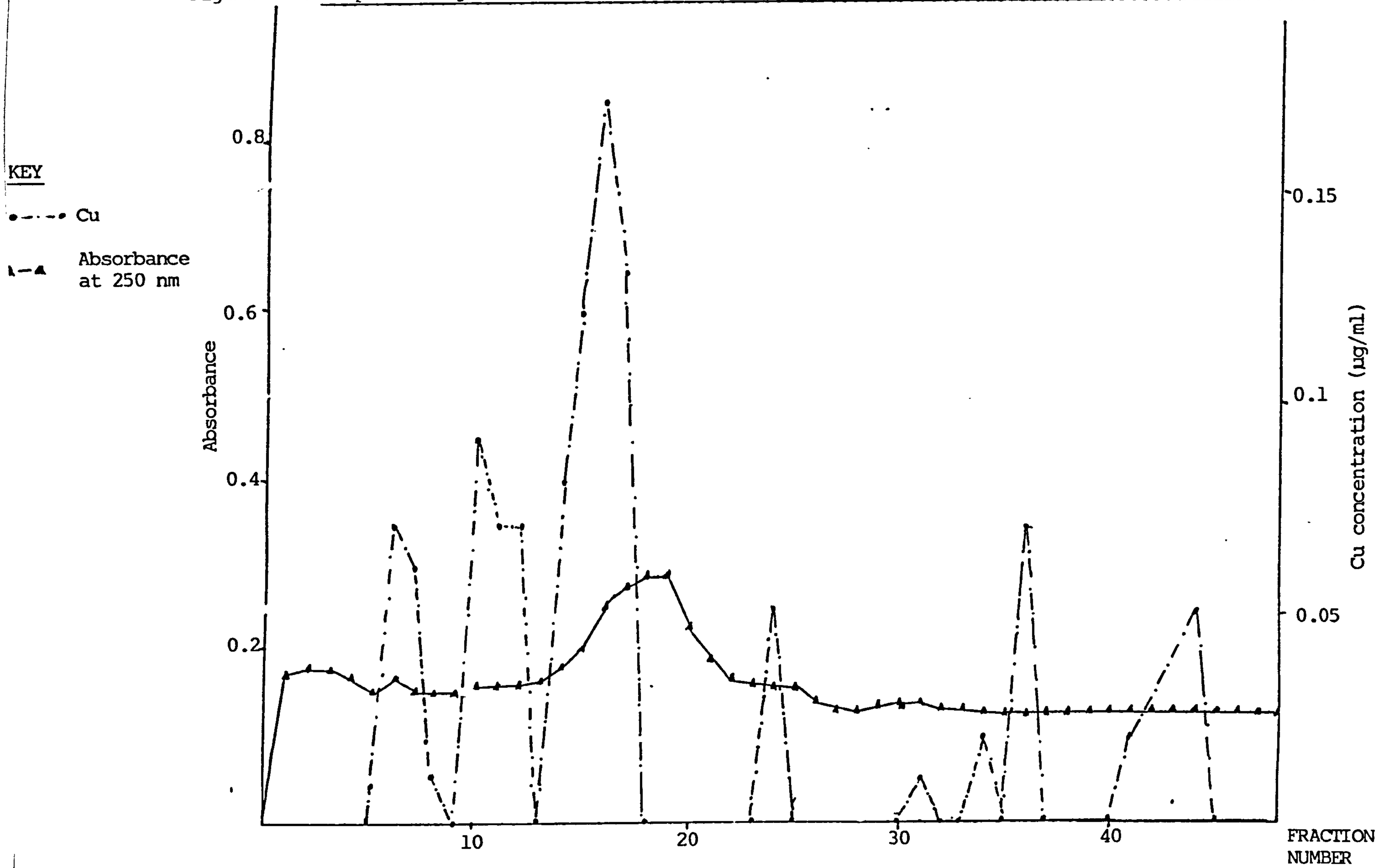


Figure 77 : DEAE-Cellulose chromatography of the Cu containing fraction of A. tenuis (Goginan) from a Sephadex G-50 column

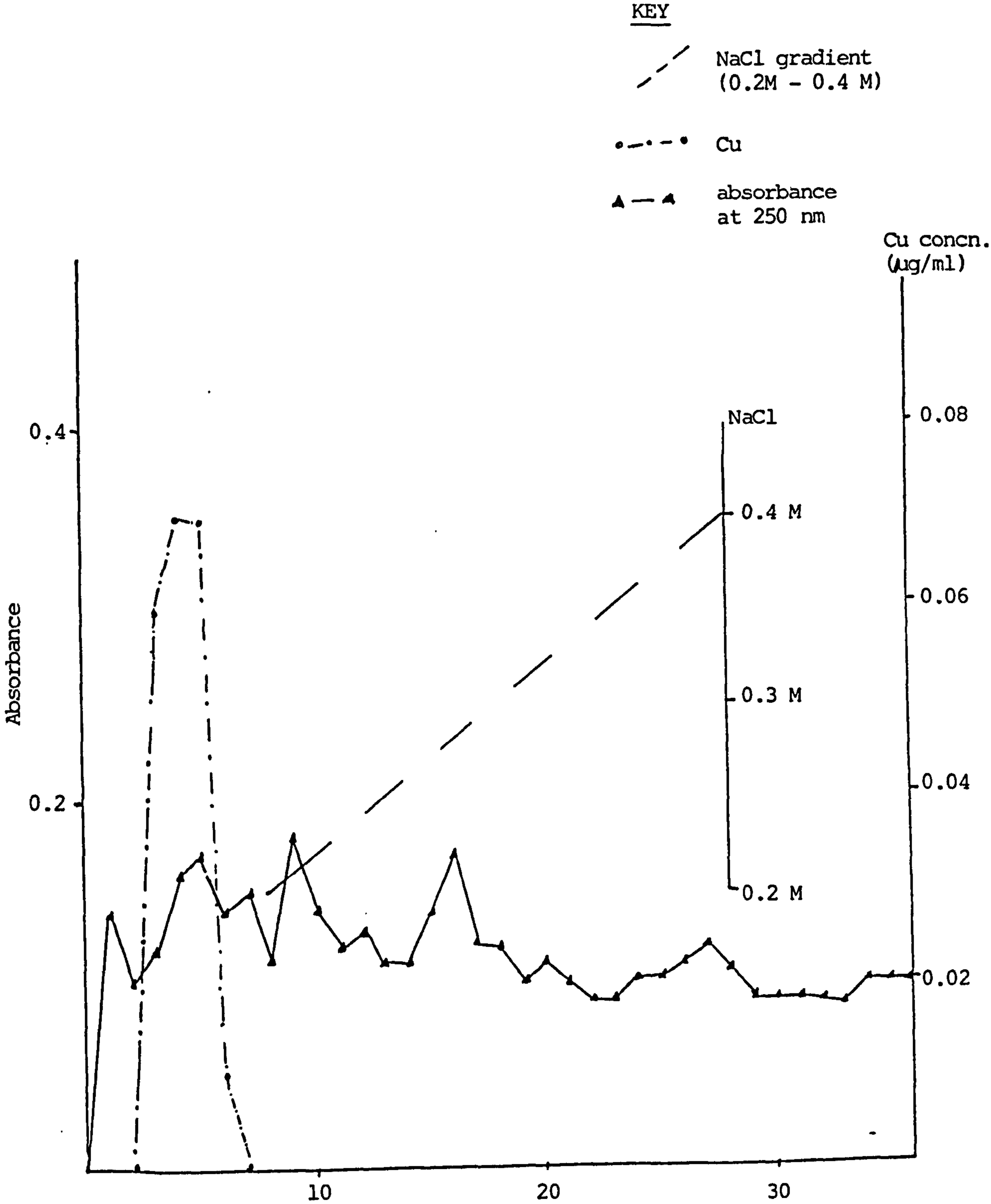


Figure 78 : Preparative gel filtration of a high speed supernatant of Silene vulgaris roots (collected from Shipham)

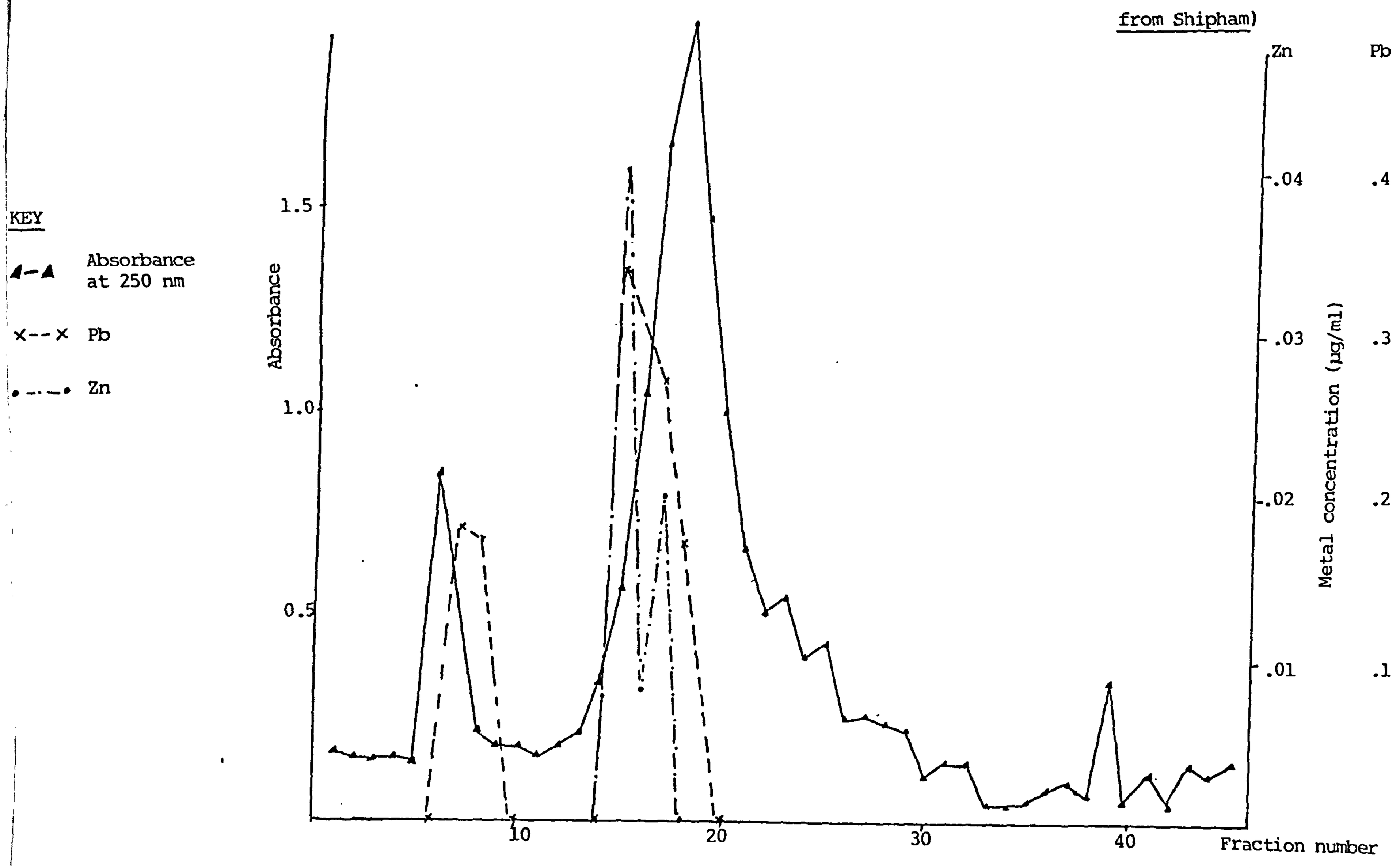


Figure 79 : DEAE-Cellulose chromatography of the Zn/Pb fraction of S. vulgaris roots from a Sephadex G-50 column

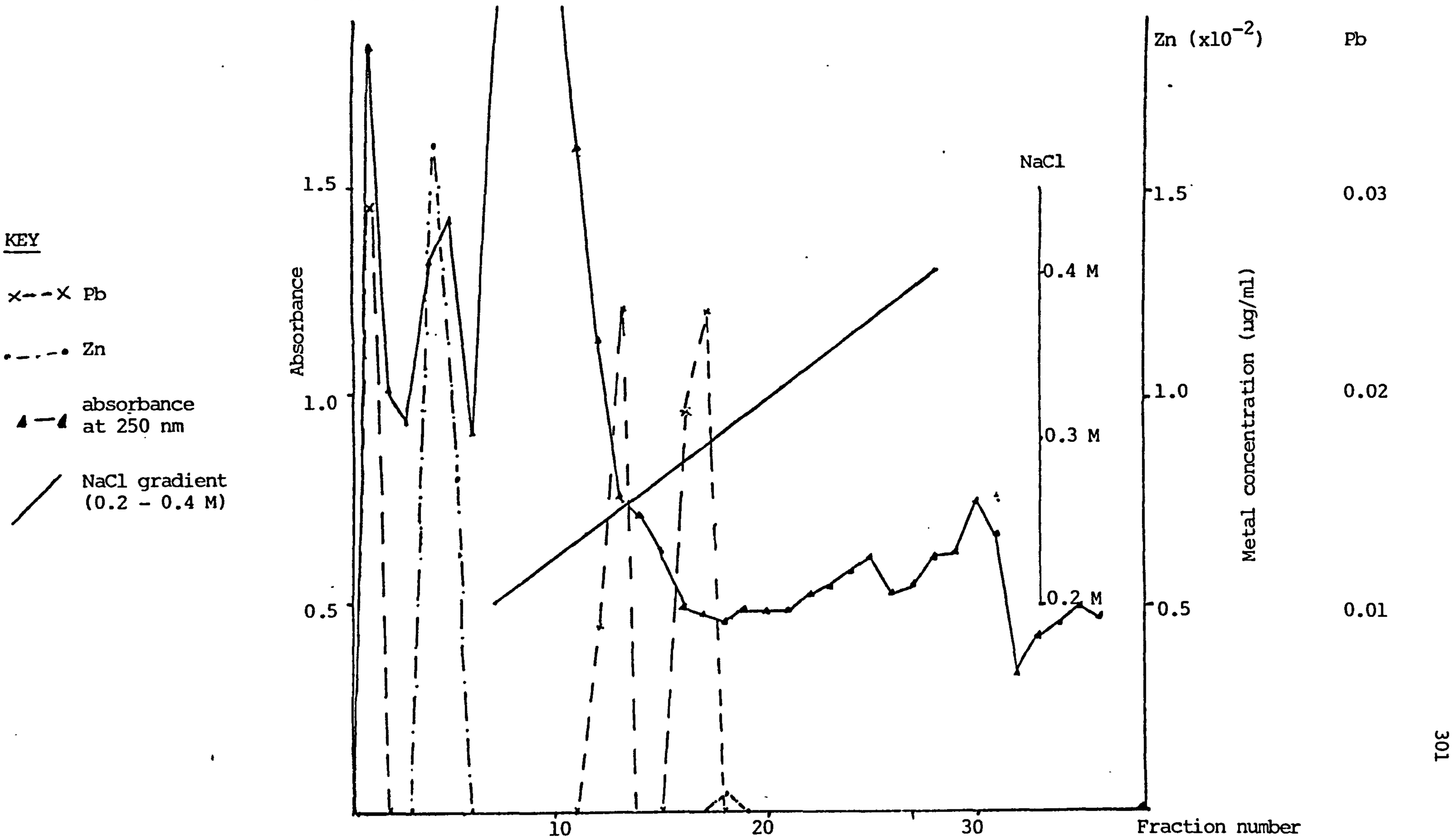


Figure 80 : Preparative gel filtration of a high speed supernatant of Thlaspi alpestre roots (collected from Charterhouse)

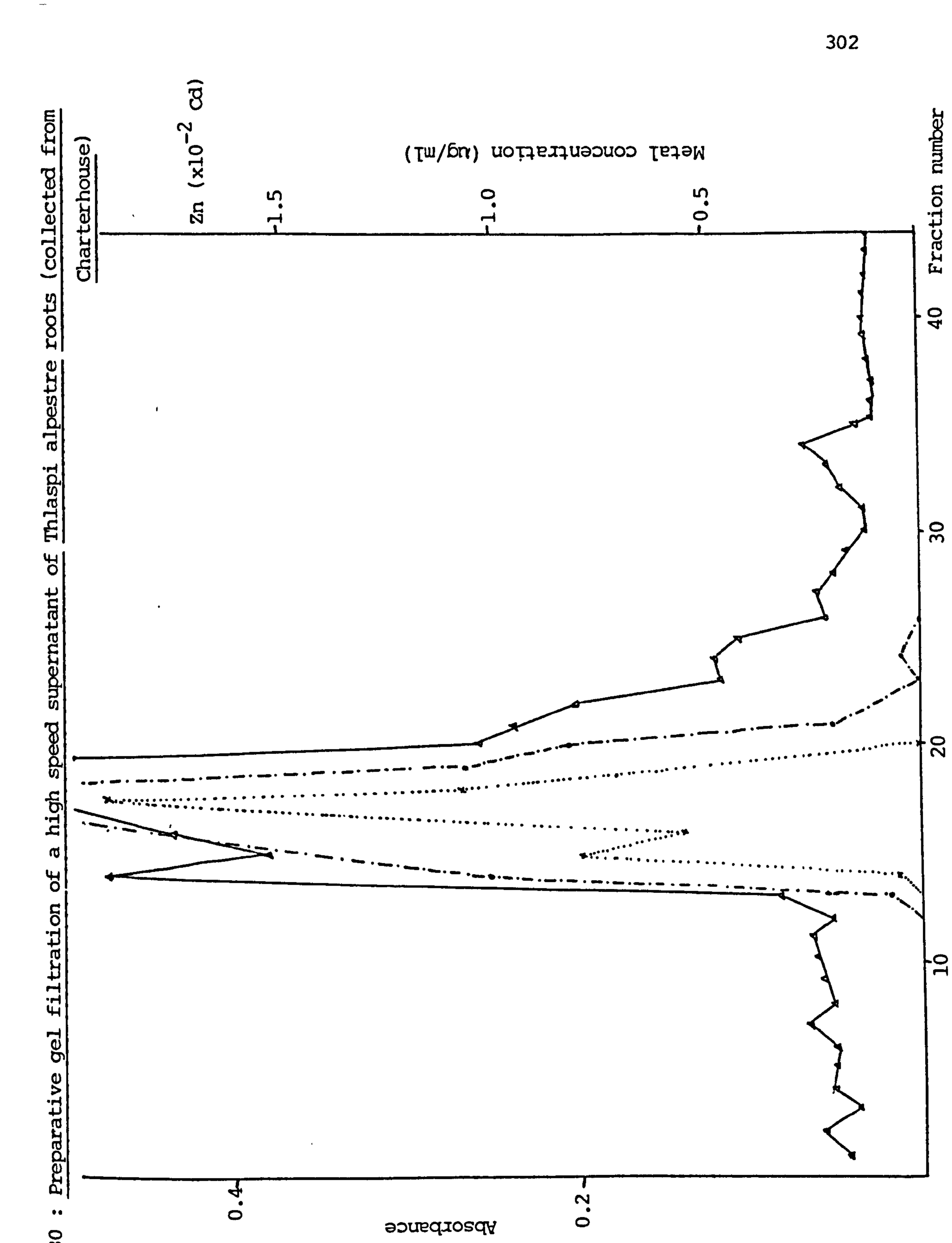
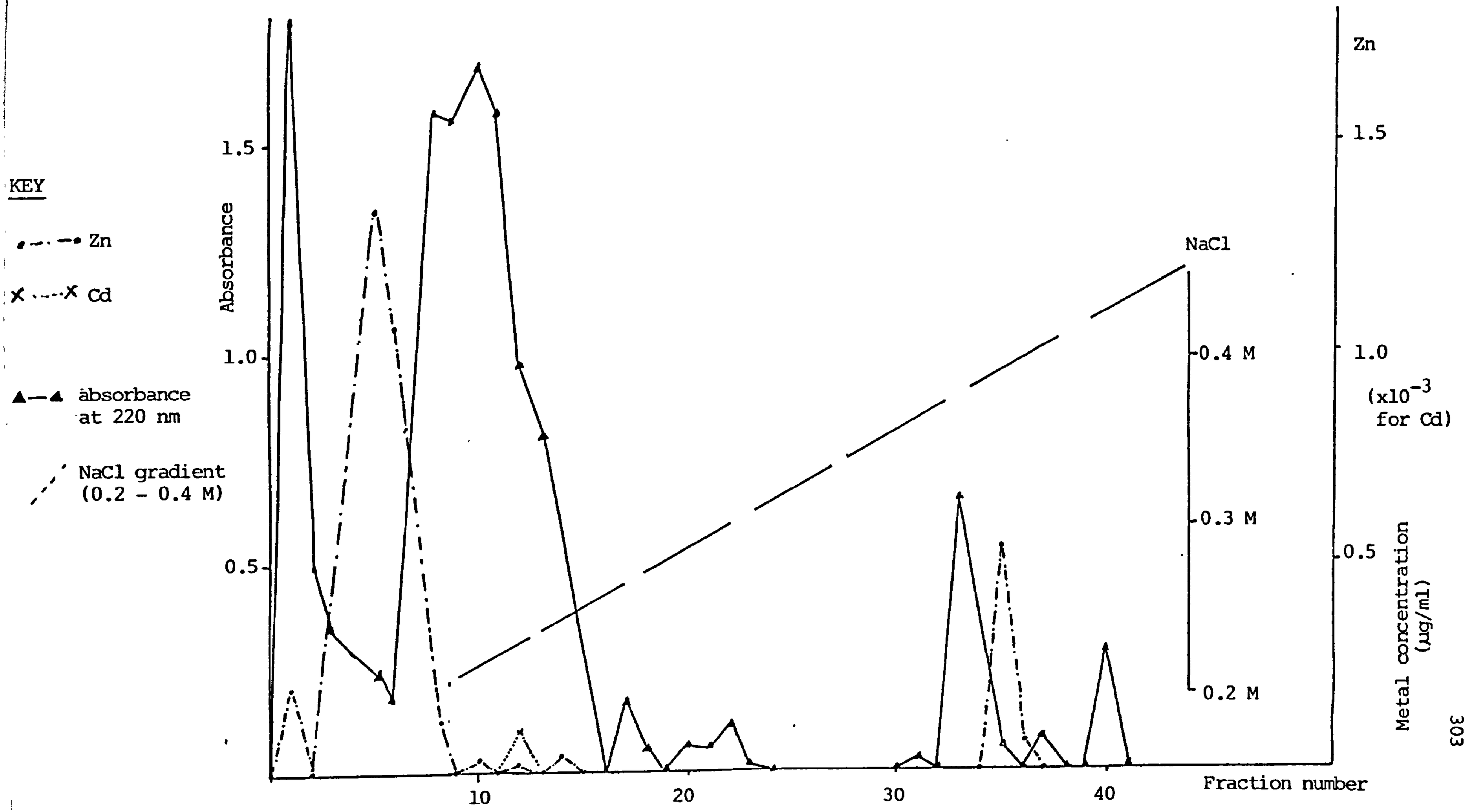


Figure 81 : DEAE-Cellulose chromatography of the Zn/Cd fraction of T. alpestre roots from a Sephadex G-50 column



d) Apparatus and Instrumental Parameters

FAAS

See Part II, Section 1.

UV Spectrophotometry

Apparatus

Unicam SP1700 Spectrophotometer

Unicam SP1803 Wavelength Selector

Unicam SP1805 Programme Controller

Unicam SP40P Automatic Sample Changer

Unicam DR10 Digital Printer

Unicam AR25 Linear Recorder

Instrumental Parameters

Unicam SP1700

Wavelength Range	200 to 300 nm

Unicam SP1805

Scan Speed	1.0 nm/s
Band Width	1.2 nm
Slit Width	0.35 mm

Unicam SP1805

Wavelengths	220, 230, 240, 250 & 280 nm

SECTION 7 : STATISTICS

7.1 Statistics for Standard Addition Method in DPASV

7.1.1 DPASV-HMDE

a) Analytical Precision

b) Reproducibility

7.1.2 DPASV-RDE

a) Analytical Precision

b) Reproducibility

7.2 Confidence Bounds and Working-Hotelling Regions

a) FAAS

b) HMDE

c) RDE

d) GFAAS

7.3 Detection Limits

a) FAAS

b) HMDE

c) RDE

d) GFAAS

a) Analytical Precision - HMDE

The analytical precision for the standard addition method was investigated by adding 20 µl of the standard solution containing 10 µg/ml of Zn, Cd, Pb and Cu to a supporting electrolyte solution (10 ml of 0.2 M ammonium citrate, pH 3.0) to which 0.2 µg of these four metals had been previously added.

Three aliquots each of 20 µl volume were added sequentially to determine the metal concentrations. The procedure was repeated 6 times. The results obtained are given in Table 93. A voltammogram of the 4 metals is given in Figure 82.

b) Reproducibility - HMDE

An aliquot of 20 µl of Zn, Cd, Pb and Cu solutions (10 µg/ml of each metal) was added to a supporting electrolyte (10 ml of 0.2 M ammonium citrate pH 3.0). The peak heights for periods up to 240s (deposition time) were measured. The spiking step was repeated 6 times for each metal studied, Tables 94 to 97 and Figures 83 to 86.

Table 93 : Mean, Standard Deviation (SD) and Standard Error (SE) of Zn, Cd, Pb and Cu concentrations found from six replicate standard additions

Subsample No.	ng M^{2+}/ml recovered			
	Zn	Cd	Pb	Cu
1	14.50	11.10	13.90	19.30
2	19.30	15.10	17.10	18.70
3	13.60	19.90	16.30	22.20
4	16.90	14.80	13.90	18.60
5	19.20	22.90	22.00	21.30
6	16.40	12.90	14.50	19.90
Mean	16.65	16.12	16.28	20.00
SD	2.14	4.05	2.83	1.33
SE	0.87	1.65	1.15	0.54

Figure 82 : Differential pulse stripping curve (HMDE) for Zn, Cd, Pb and Cu in 0.2 M ammonium citrate, pH 3

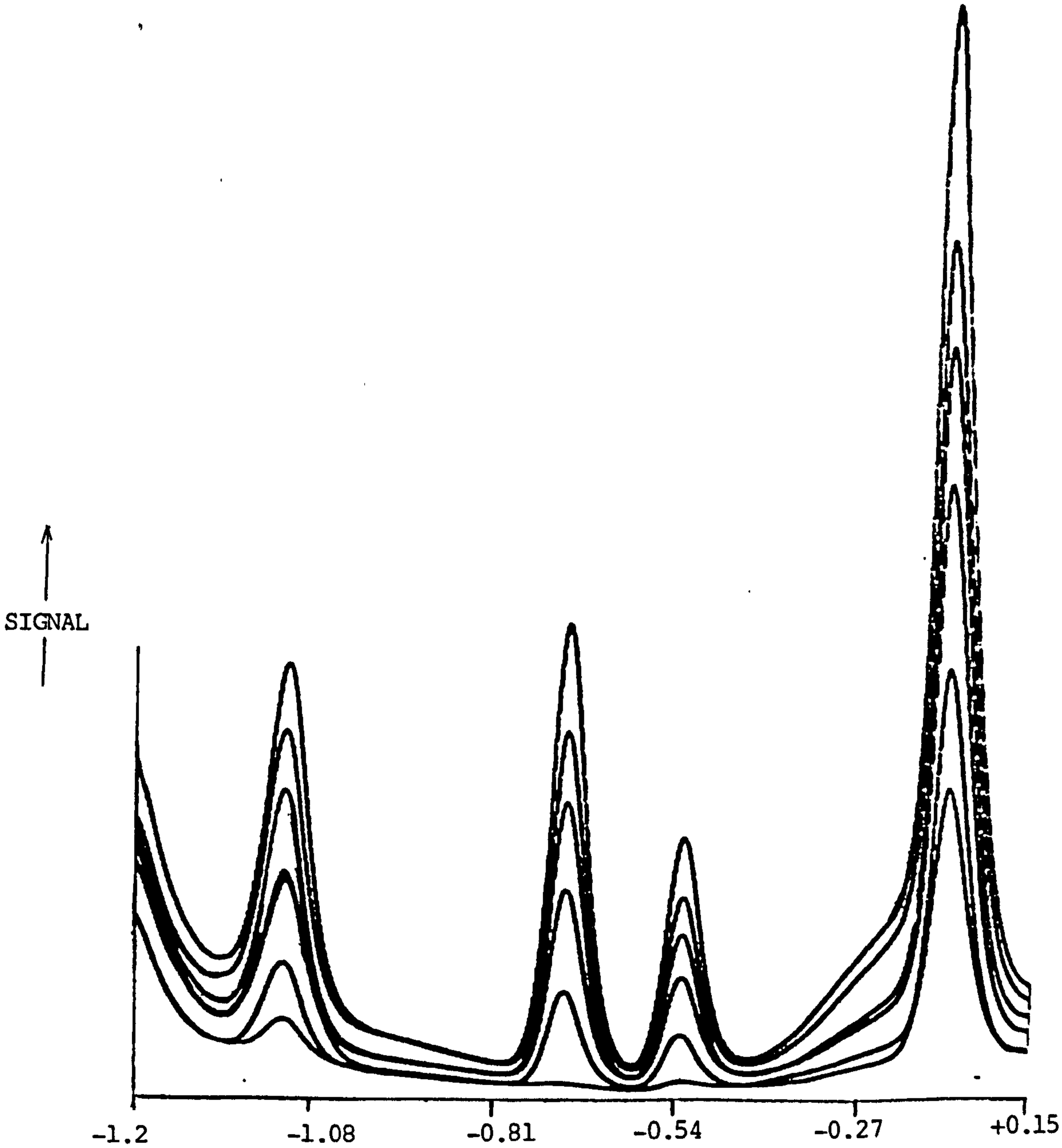


Table 94 : Effect of deposition time on Zn peak height

	Peak Height (cm)				
Subsample No.	40s	80s	120s	180s	240s
1	0.12	0.17	0.19	0.24	0.28
2	0.12	0.17	0.21	0.26	0.33
3	0.11	0.19	0.20	0.24	0.34
4	0.06	0.14	0.19	0.23	0.28
5	0.16	0.23	0.29	0.38	0.45
6	0.17	0.26	0.25	0.39	0.47
Mean	0.12	0.19	0.22	0.29	0.36
SD	0.04	0.04	0.04	0.07	0.08
SE	0.01	0.02	0.02	0.03	0.03

Table 95 : Effect of deposition time on Cd peak height

	Peak Height (cm)				
Subsample No.	40s	80s	120s	180s	240s
1	0.12	0.22	0.31	0.45	0.57
2	0.12	0.21	0.31	0.42	0.58
3	0.12	0.22	0.31	0.45	0.56
4	0.12	0.22	0.32	0.46	0.61
5	0.14	0.34	0.37	0.45	0.66
6	0.16	0.36	0.32	0.44	0.59
Mean	0.13	0.26	0.32	0.45	0.60
SD	0.02	0.06	0.02	0.01	0.03
SE	0.01	0.03	0.01	0.01	0.01

Table 96 : Effect of deposition times on Pb peak height

	Peak Height (cm)				
Subsample No.	40s	80s	120s	180s	240s
1	0.12	0.17	0.18	0.27	0.31
2	0.09	0.14	0.18	0.26	0.34
3	0.06	0.12	0.16	0.29	0.32
4	0.07	0.12	0.18	0.26	0.38
5	0.06	0.11	0.16	0.24	0.30
6	0.08	0.11	0.16	0.24	0.32
Mean	0.08	0.13	0.17	0.26	0.33
SD	0.02	0.02	0.01	0.02	0.03
SE	0.01	0.01	0.005	0.01	0.01

Table 97 : Effect of deposition times on Cu peak height

	Peak Height (cm)				
Subsample No.	40s	80s	120s	180s	240s
1	0.30	0.57	0.83	1.20	1.58
2	0.22	0.39	0.56	0.81	1.06
3	0.23	0.42	0.63	1.09	1.26
4	0.26	0.47	0.69	0.99	1.30
5	0.25	0.46	0.67	1.00	1.32
6	0.25	0.46	0.69	1.04	1.28
Mean	0.25	0.46	0.68	1.02	1.30
SD	0.03	0.06	0.08	0.12	0.15
SE	0.01	0.02	0.03	0.05	0.06

Figure 83 : Dependence of Zn peak height on deposition time (for HMDE)

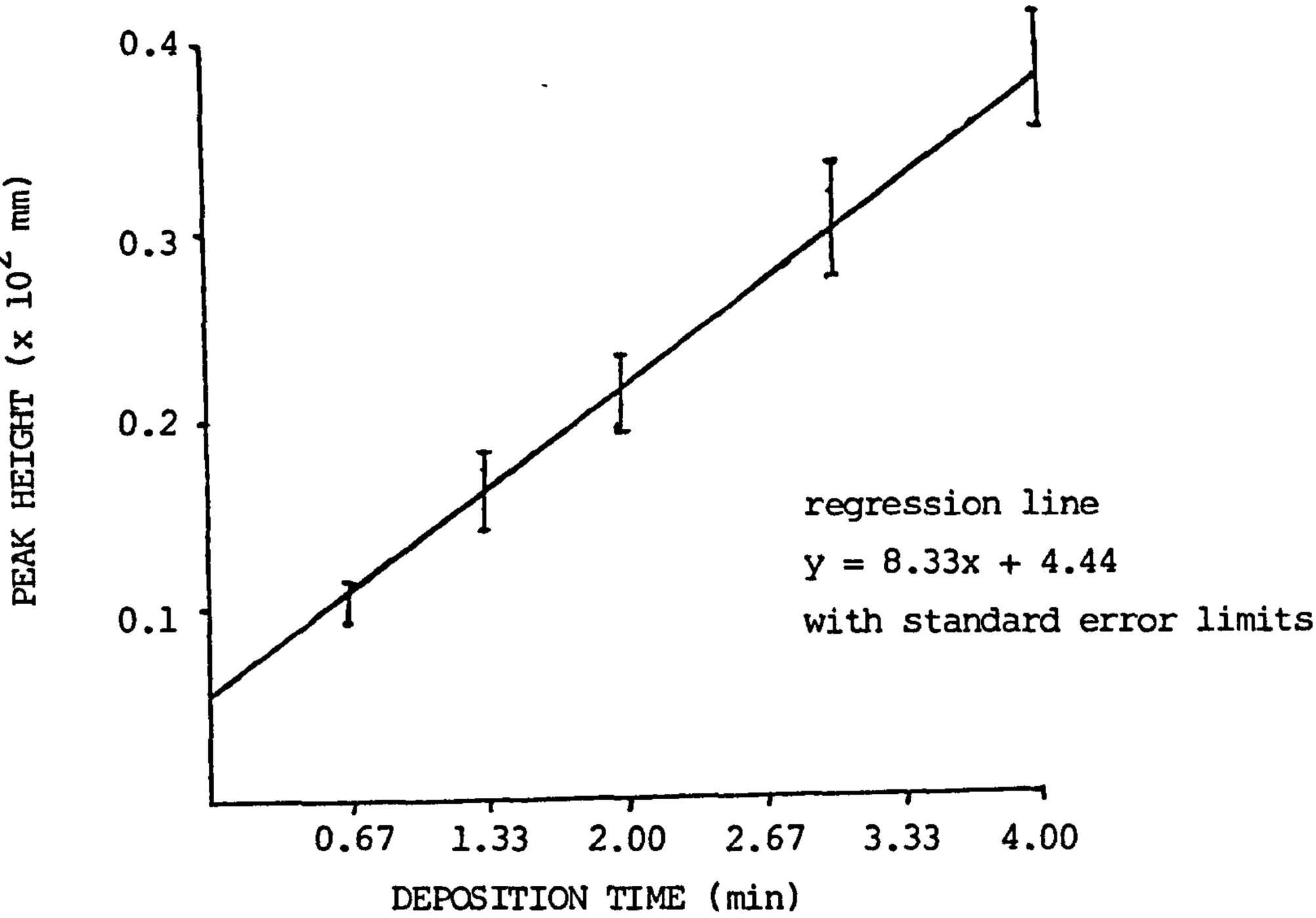


Figure 84 : Dependence of Cd peak height on deposition time (for HMDE)

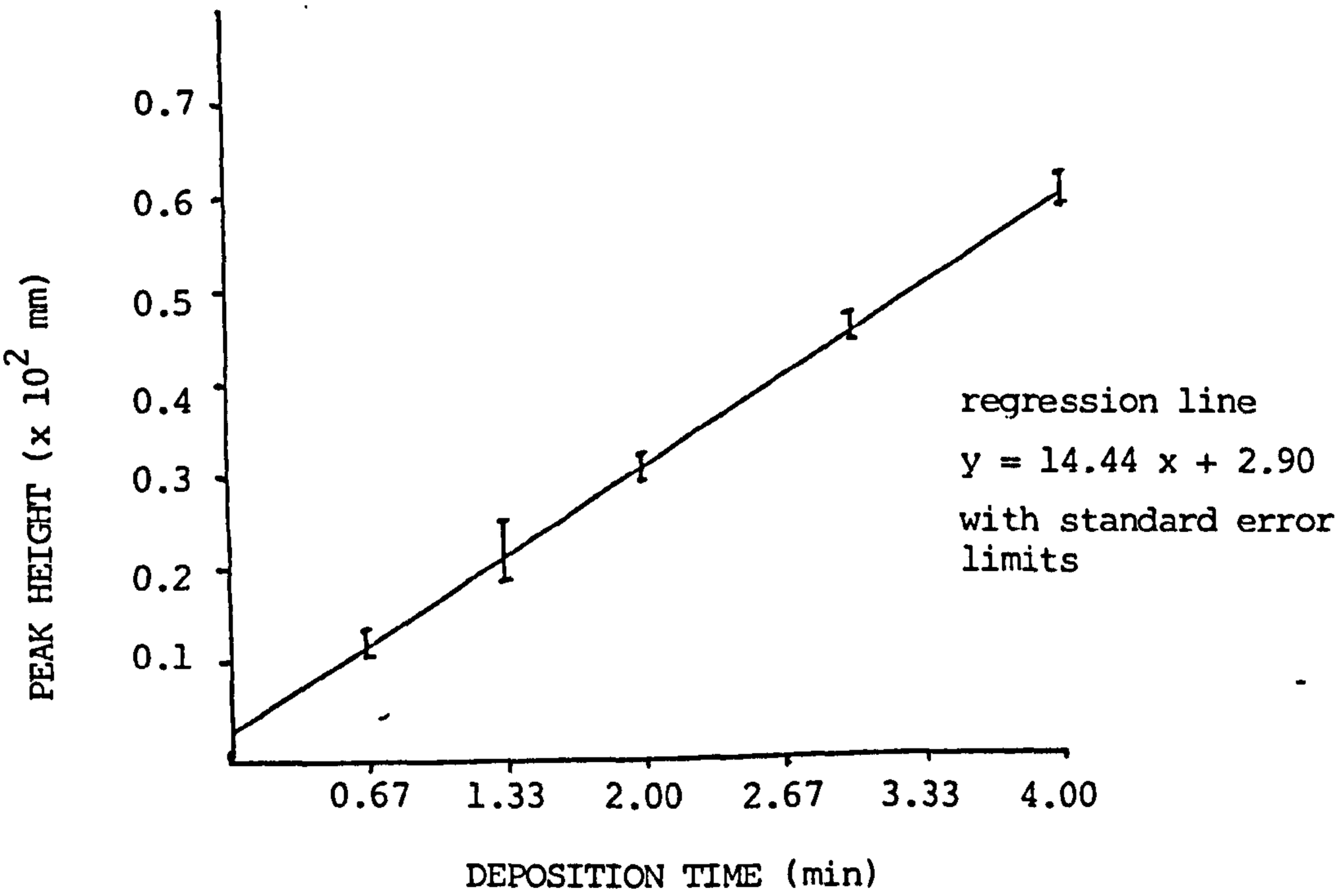


Figure 85 : Dependence of Pb peak height on deposition time (for HMDE)

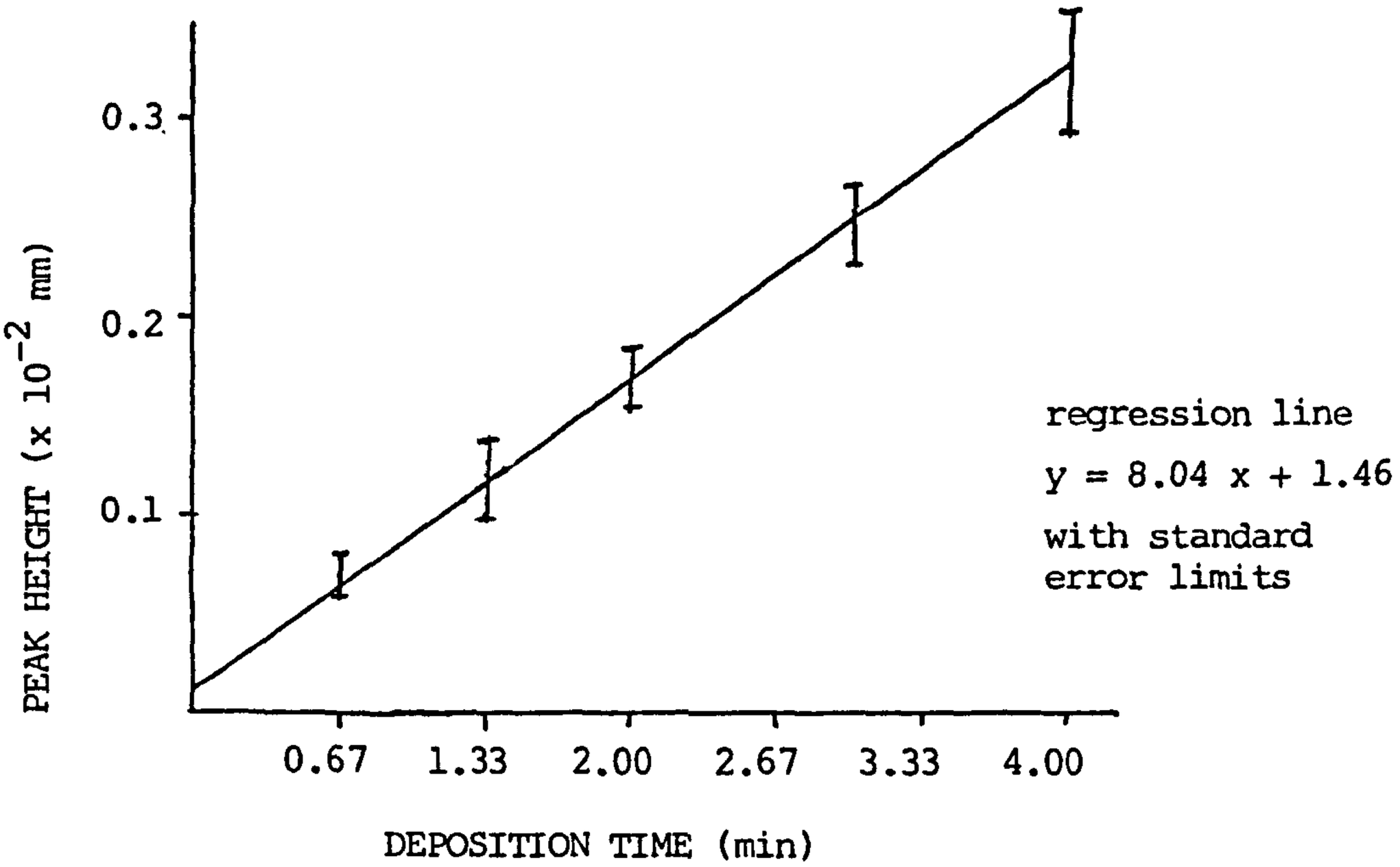
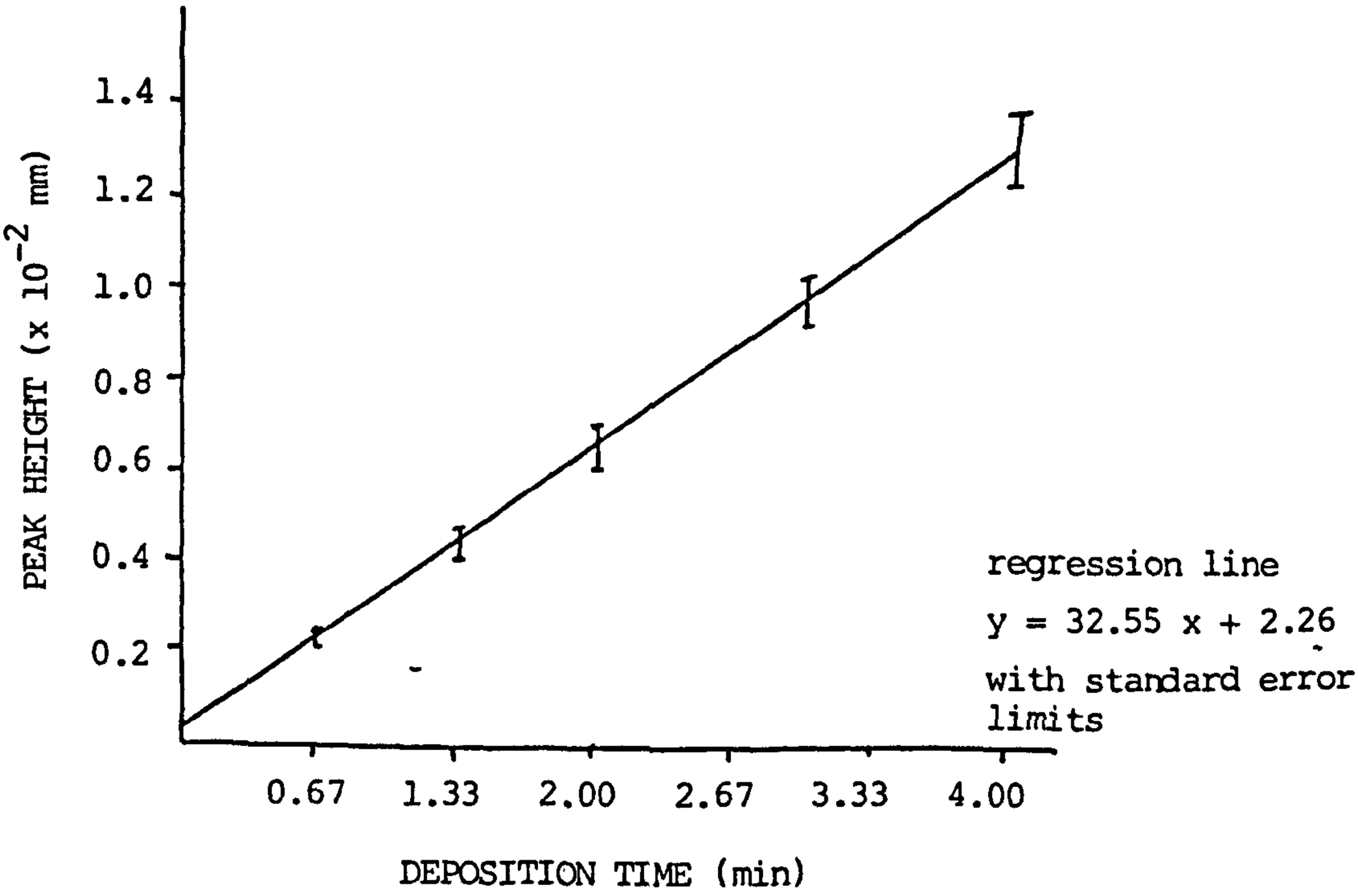


Figure 86 : Dependence of Cu peak height on deposition time (for HMDE)



a) Analytical Precision - RDE

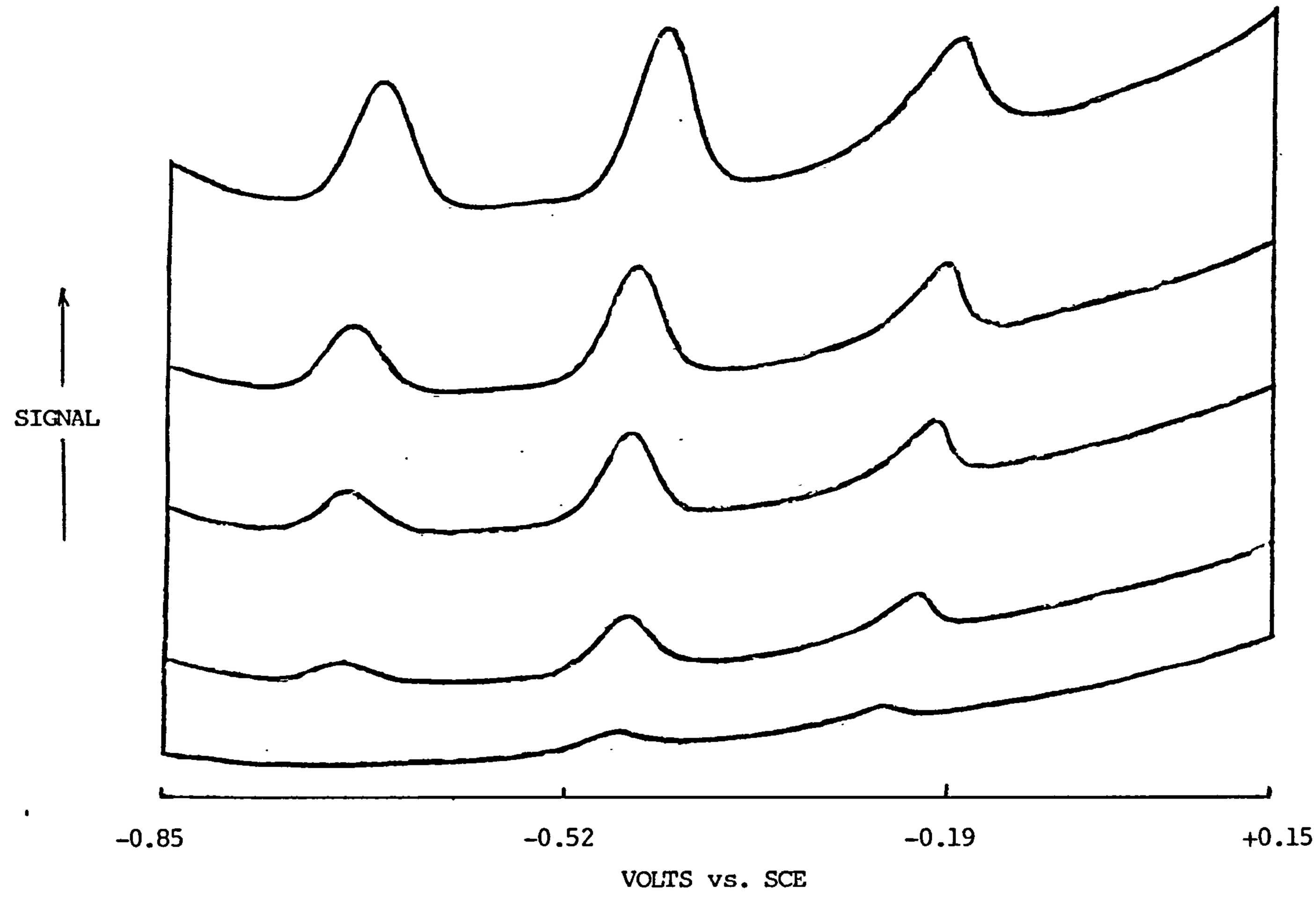
The analytical precision for the standard addition method was investigated by adding 20 µl of the standard solution containing 10 µg/ml of Cd, Pb, and Cu to a supporting electrolyte (15 ml of 1 M ammonium chloride, 0.1 M citric acid and 0.025 M ascorbic acid pH 2.5) to which 0.2 µg of these metals had been previously added.

Three aliquots each of 20 µl volume were added to determine the metal concentrations. The procedure was repeated 6 times. The results obtained are given in Table 98. A voltammogram of the 3 metals is given in Figure 87.

Table 98 : Mean, Standard Deviation (SD) and Standard Error (SE) of Cd, Pb and Cu concentrations found from six replicate standard additions

	ng M^{2+}/ml Recovered		
Subsample No.	Cd	Pb	Cu
1	15.0	15.0	20.0
2	12.0	18.0	18.0
3	15.0	16.0	21.0
4	17.0	11.0	18.0
5	14.0	15.0	20.0
6	18.0	16.0	21.0
Mean	15.2	15.2	19.67
SD	1.95	2.11	1.25
SE	0.80	0.86	0.51

Figure 87 : Differential pulse stripping curve (RDE) for Cd, Pb and Cu in (1 M ammonium chloride; 0.1 M citric acid and 0.025 M ascorbic acid) pH 2.5



b) Reproducibility - RDE

An aliquot of 20 ul of Cd, Pb, and Cu solutions (10 µg/ml of each metal) was added to a supporting electrolyte (15 ml of 1 M ammonium chloride, 0.1 M citric acid and 0.025 M ascorbic acid pH 2.5). The peak heights for periods up to 300s (deposition time) were measured. The spiking step was repeated 6 times for each metal studied (Tables 99 to 101 and Figures 85 to 90).

Table 99 : Effect of deposition time on Cd peak height

	Peak Height (cm)					
Subsample No.	30s	60s	120s	180s	240s	300s
1	0.7	1.1	5.3	6.9	7.5	11.0
2	1.0	1.2	5.1	6.6	6.9	9.8
3	0.7	0.7	5.2	6.7	7.1	10.9
4	1.0	0.8	4.9	6.6	7.7	10.8
5	0.7	1.0	4.8	7.1	7.6	10.8
6	0.5	0.8	5.1	6.5	7.5	9.9
Mean	0.77	0.93	5.07	6.73	7.38	10.53
SD	0.18	0.18	0.17	0.21	0.29	0.49
SE	0.07	0.07	0.07	0.08	0.12	0.20

Table 100 : Effect of deposition time on Pb peak height

	Peak Height (cm)					
Subsample No.	30s	60s	120s	180s	240s	300s
1	0.9	1.1	4.4	5.4	6.7	9.1
2	0.7	1.2	4.3	5.9	6.3	9.2
3	1.3	1.6	4.4	5.7	6.6	8.9
4	1.1	1.5	4.5	5.6	6.1	9.9
5	0.9	1.5	4.2	5.8	6.8	9.4
6	0.9	1.2	4.1	5.4	6.5	9.6
Mean	0.97	1.35	4.32	5.63	6.50	9.35
SD	0.19	0.19	0.13	0.19	0.24	0.33
SE	0.08	0.08	0.05	0.08	0.10	0.13

Table 101 : Effect of deposition time on Cu peak height

	Peak Height (cm)					
Subsample No.	30s	60s	120s	180s	240s	300s
1	0.9	1.1	2.8	3.9	5.1	8.9
2	1.0	1.2	2.7	4.3	5.5	9.3
3	0.7	1.0	2.8	4.1	4.9	8.7
4	1.0	0.9	2.5	3.8	5.2	8.4
5	0.9	1.3	2.9	3.7	4.8	9.1
6	0.5	0.7	2.4	4.0	5.3	8.6
Mean	0.83	1.03	2.68	3.97	5.13	8.83
SD	0.18	0.20	0.18	0.20	0.24	0.30
SE	0.07	0.08	0.07	0.08	0.10	0.12

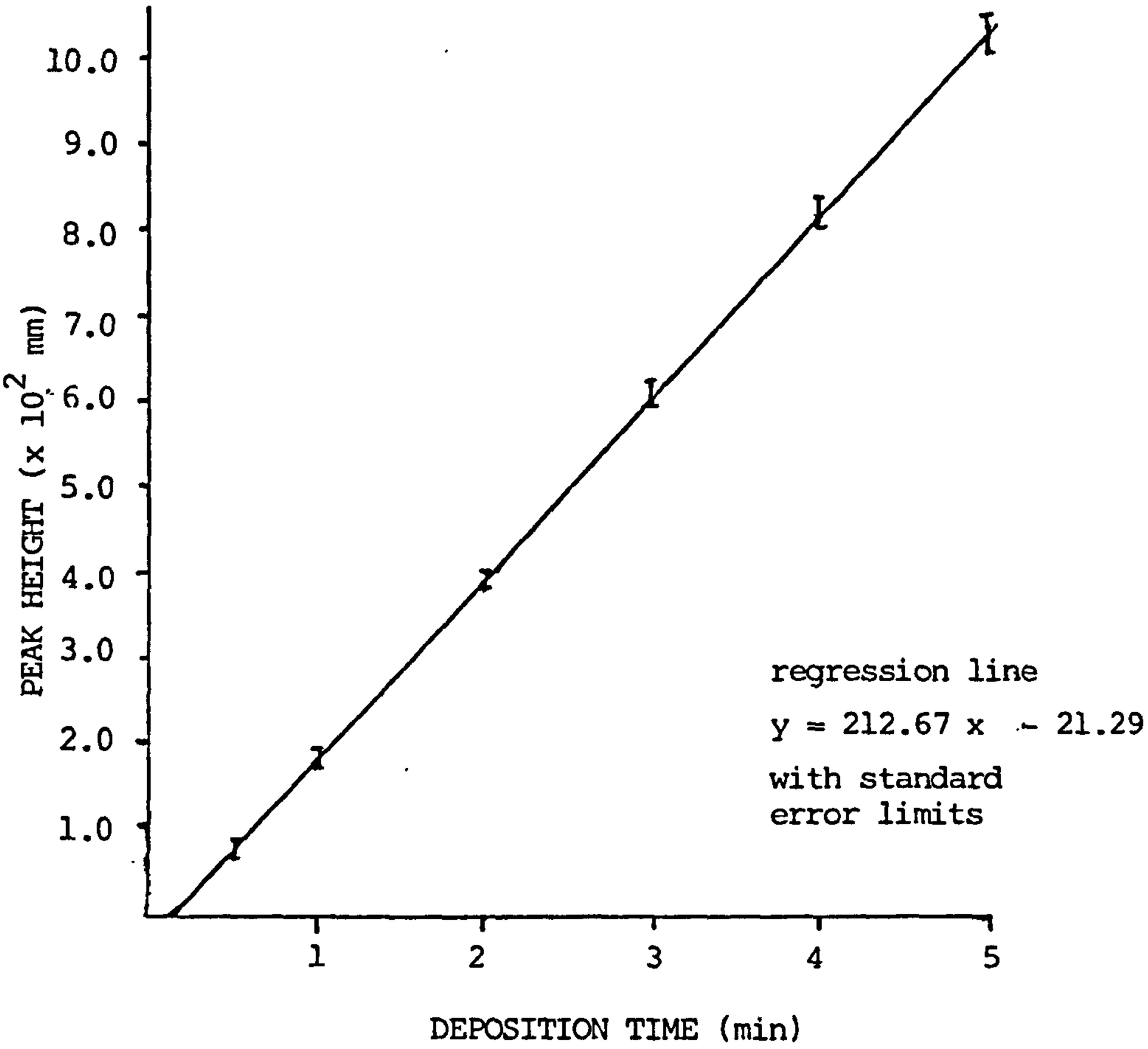


Figure 88 : Dependence of Cd peak height on deposition time (for RDE)

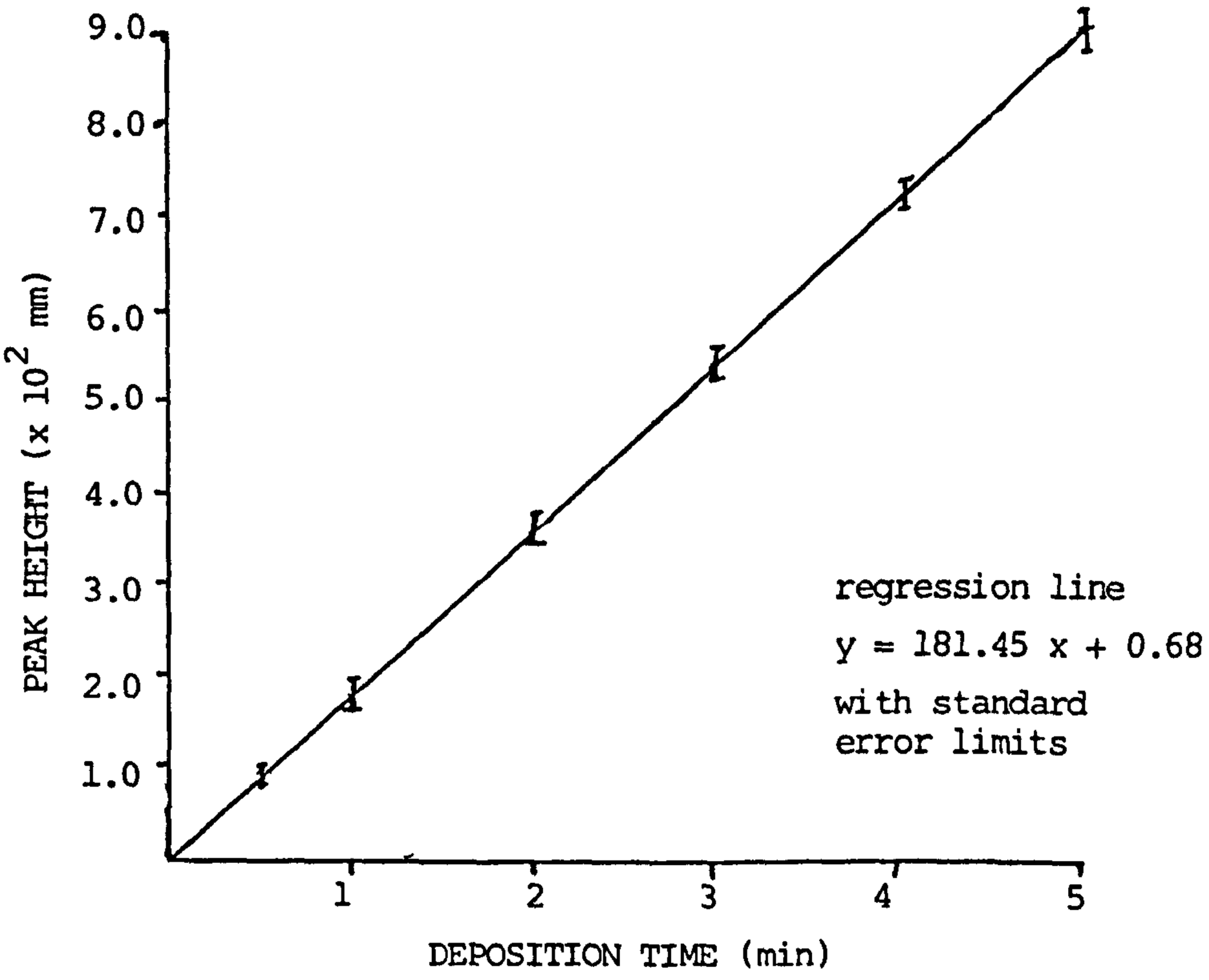
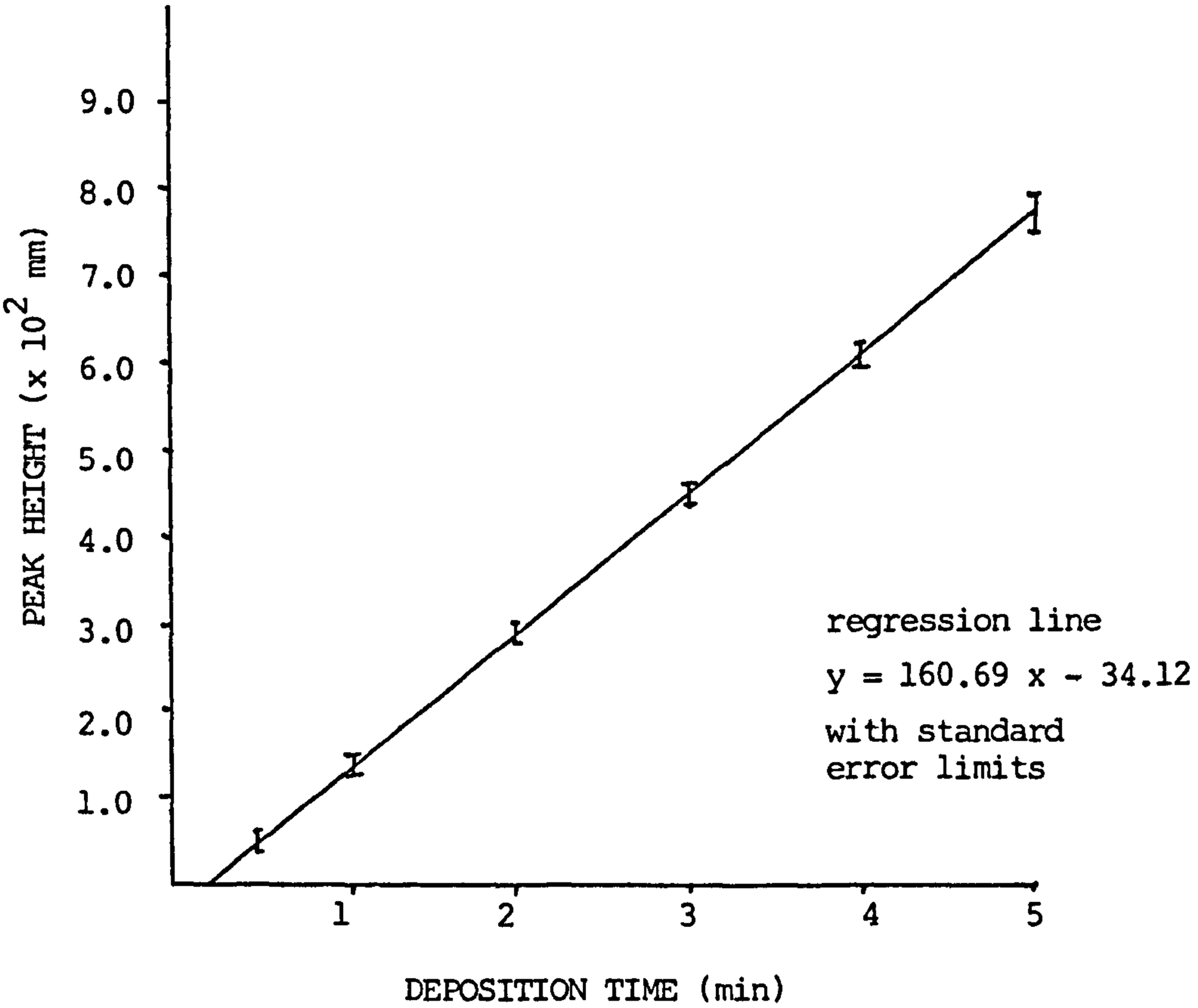


Figure 89 : Dependence of Pb peak height on deposition time (RDE)

Figure 90 : Dependence of Cu peak height on deposition time (for RDE)



Calibration of Zn line for FAAS (290,291)

Concn.(µg/ml)	DIGITAL READOUT	$x=X-\bar{X}$	$y=Y-\bar{Y}$	x^2	y^2
0.25	2.3	-0.75	-6.0667	0.5625	36.8048
0.50	4.3	-0.50	-4.0667	0.2500	16.5380
0.75	6.4	-0.25	-1.9667	0.0625	3.8679
1.00	8.7	0.00	0.3333	0.0000	0.1111
1.50	12.1	0.50	3.7333	0.2500	13.9375
2.00	16.4	1.00	8.0333	1.0000	64.5339
$\sum X=6$	$\sum Y=50.2$	$\sum x=0.00$	$\sum y=0.0002$	$\sum x^2=2.125$	$\sum y^2=135.7933$
$\bar{X}=1$	$\bar{Y}=8.3667$	$\bar{x}=0.00$	$\bar{y}=0.00003$		

xy
4.5500
2.0334
0.4917
0.0000
1.86665
8.0333
$\sum xy=16.9751$

$$\text{Slope } b = \frac{\sum xy}{\sum x^2} = \frac{16.9751}{2.125} = \underline{7.9883}$$

$$\text{intercept } a = \bar{Y} - b\bar{X} = 8.3667 - 7.9883 \times 1.0 = \underline{0.3784}$$

$$\text{Variance } s^2 = \frac{(\sum y^2) - (b \sum xy)}{(n-2)} = \frac{135.7932 - (7.9883 \times 16.9751)}{4}$$

$$s^2 = \underline{0.04775}$$

$\hat{y}$ = predicted value for true response for any given value of x

$$\hat{y} = a + bx$$

$$\underline{\hat{y} = 0.3784 + 7.9883x} = \text{prediction equation.}$$

$\hat{y}$ values are given in Table 102.

95% Confidence bounds for 90% of observations :-

$$y = a + bx \pm (A + Zp\ B)$$

where, $A = \left[2\ F_{2,\nu,1-\alpha/2}\ \left(\frac{1}{n} + \frac{(x - \bar{x})^2}{\sum x^2}\right)\ s^2\right]^{\frac{1}{2}}$

$$B = \left[\nu s^2 / \chi^2_{\nu/1-\frac{\alpha}{2}}\right]^{\frac{1}{2}}$$

$F_{2,4,0.025} = 10.65$ $\qquad$ $Zp = 1.29$

$$\chi^2_{4,0.975} = 0.484$$

$$y_1 = 0.3784 + 7.9883\ X_1 \pm \left\{\left[2(10.65)\left(\frac{1}{6} + \frac{\left(X_1 - 1\right)^2}{2.125}\right) \times 0.04775\right]^{\frac{1}{2}} + 1.29\left[\frac{4(0.04775)}{0.484}\right]^{\frac{1}{2}}\right\}$$

$$= 2.37548 \pm 0.66237 + 0.81037$$

$$= 2.37548 \pm 1.47374$$

$$\underline{y_1 = 3.84822 \text{ or } 0.90274}$$

$$y_2 = 0.3784 + 7.9883 \times 0.50 \pm \left\{\left[2.13\left(0.166667 + \frac{(-0.5)^2}{2.125}\right) 0.04775\right]^{\frac{1}{2}} + 0.80867\right\}$$

$$= 4.37255 \pm 0.53774 + 0.81037$$

$$\underline{y_2 = 5.72066 \text{ or } 3.02444}$$

$$y_3 = 0.3784 + 7.9883 \times 0.75 \pm \left\{ \left[21.3 \left(0.166667 + \frac{(0.75 - 1)^2}{2.125} \right) \times 0.04775 \right]^{\frac{1}{2}} + 0.81037 \right\}$$

$$= 6.36963 \pm 0.44657 + 0.81037$$

$$= 6.36963 \pm 1.25694$$

$$\underline{y_3 = 7.6266 \quad \text{or} \quad 5.11269}$$

$$y_4 = 0.3784 + 7.9883 \times 1 \pm \left\{ \left[21.3 \left(0.16667 + \frac{(0)^2}{2.125} \right) \times 0.04775 \right]^{\frac{1}{2}} + 0.81037 \right\}$$

$$= 8.3667 \pm (0.41172 + 0.81037)$$

$$= 8.3667 \pm 1.22209$$

$$\underline{y_4 = 9.58879 \quad \text{or} \quad 7.14461}$$

$$y_5 = 0.3784 + 7.9883 \times 1.5 \pm \left\{ \left[21.3 \left(0.16667 + \frac{(0.5)^2}{2.125} \right) \times 0.04775 \right]^{\frac{1}{2}} + 0.81037 \right\}$$

$$= 12.36085 \pm 0.53774 + 0.81037$$

$$= 12.36085 \pm 1.34811$$

$$\underline{y_5 = 13.70896 \quad \text{or} \quad 11.01274}$$

$$y_6 = 0.3784 + 7.9883 \times 2 \pm \left\{ \left[21.3 \left(0.16667 + \frac{(1)^2}{2.125} \right) \times 0.04775 \right]^{\frac{1}{2}} + 0.81037 \right\}$$

$$= 16.355 + 0.805069 + 0.81037$$

$$= 16.355 \pm 1.615439$$

$$\underline{y_6 = 17.97044 \quad \text{or} \quad 14.73956}$$

Working-Hotelling region is given by

$$y = a + bX \pm \left\{ 2F_{2,\nu,\alpha} \left[\frac{1}{n} + \frac{(x - \bar{x})^2}{\sum x^2} \right] s^2 \right\}^{\frac{1}{2}}$$

$$y_1 = 0.3784 + 7.9883 \times 0.25 \pm \left\{ 2 \times 6.94 \left(\frac{1}{6} + \frac{(0.25 - 1)^2}{2.125} \right) \times 0.04775 \right\}^{\frac{1}{2}}$$

$$= 2.375475 \pm 0.534697$$

$$= \underline{1.840778 \text{ or } 2.91017}$$

$$y_2 = 0.3784 + 7.9883 \times 0.5 \pm \left\{ 0.66277 \left(0.1666667 + \frac{(0.5)^2}{2.125} \right) \right\}^{\frac{1}{2}}$$

$$= 4.37255 \pm 0.434091$$

$$= \underline{3.93846 \text{ or } 4.806641}$$

$$y_3 = 0.3784 + 7.9883 \times 0.75 \pm \left\{ 0.66277 \left(0.166666 + \frac{(0.75 - 1)^2}{2.125} \right) \right\}^{\frac{1}{2}}$$

$$= 6.369625 \pm 0.360493$$

$$= \underline{6.00913 \text{ or } 6.75674}$$

$$y_4 = 0.3784 + 7.9883 \times 1 \pm \left\{ 0.66277 \left(0.166666 + \frac{(1 - 1)^2}{2.125} \right) \right\}^{\frac{1}{2}}$$

$$= 8.3667 \pm 0.332358$$

$$= \underline{8.03434 \text{ or } 8.69906}$$

$$y_5 = 0.3784 + 7.9883 \times 1.5 \pm \left\{ 0.6627 \left(0.166666 + \frac{(0.5)^2}{2.125} \right) \right\}^{\frac{1}{2}}$$

$$= 12.36085 \pm 0.434091$$

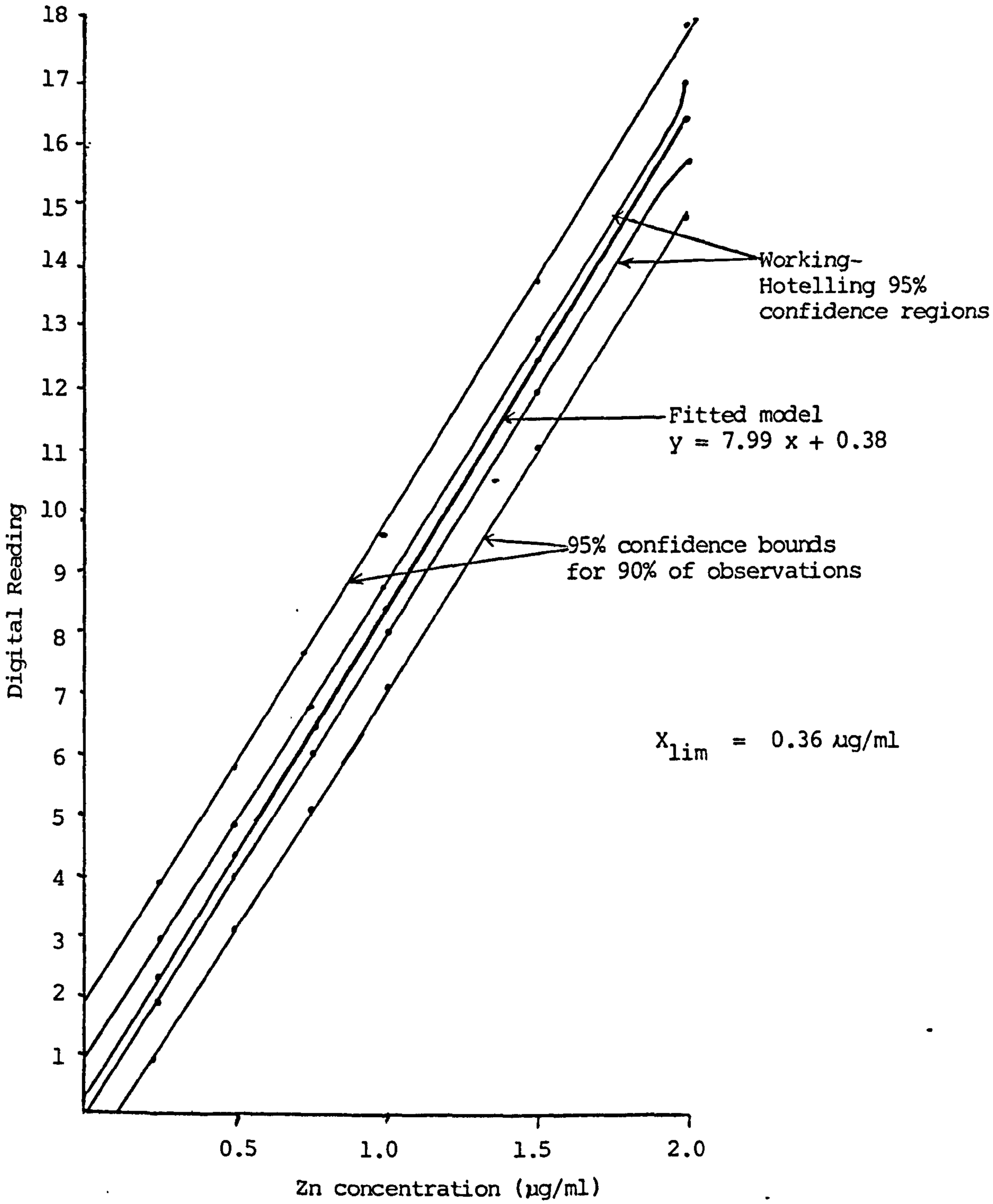
$$= \underline{11.926759 \text{ or } 12.79494}$$

$$y_6 = 0.3784 + 7.9883 \times 2 \pm \left\{ 0.66277 \left(0.166666 + \frac{(1)^2}{2.125} \right) \right\}^{\frac{1}{2}}$$

$$= 16.3550 \pm 0.649887$$

$$= \underline{15.7051 \text{ or } 17.00489}$$

Figure 91 : Fitted line for Zn by FAAS with its Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations y



Determination of the lower limit of detection X_{LD} (292)

The limit of detection was calculated by comparing two prediction limits. The upper prediction limit of the blank was calculated and the lower prediction limits on areas for given concentrations were calculated. The lower prediction limits were compared with the upper prediction limit on the blank until a lower prediction limit was found which was greater than or equal to the upper prediction limit on the blank.

Y_{UB}, a 99% upper prediction limit on the blank, was calculated by using the following equation :-

$$Y_{UB} = a + bx + \left\{ t.s \left[\sqrt{1 + \frac{1}{n} + \frac{(\bar{X})^2}{\sum x^2}} \right] \right\}$$

For a 99% confidence ($\alpha = 0.01$) and n-2 degrees of freedom :-

$$Y_{UB} = 0.3784 + 7.9883 \times 0 + \left\{ 4.604 \times 0.21852 \left[\sqrt{1 + \frac{1}{6} + \frac{(1)^2}{2.125}} \right] \right\}$$

$$Y_{UB} = \underline{2.0255713}$$

Y_L, the 99% lower prediction limit on the expected area at a given concentration, was calculated by using the equation :-

$$Y_L = a + bx - \left\{ t.s \left[\sqrt{1 + \frac{1}{6} + \frac{(Xo - \bar{X})^2}{\sum x^2}} \right] \right\}$$

$$Y_L = 0.3784 + 7.9883x - \left\{ 1.0060568 \left[\sqrt{1 + \frac{1}{6} + \frac{(Xo - 1)^2}{2.125}} \right] \right\}$$

Values for Xo were substituted into the above equation. The lowest value of X which gave a value of Y_L exceeding or equalling Y_{UB} was the lower limit of detection X_{LD}. (313)

i) For x = 0.25

Y_L = 2.34915 - 1.203646 = 1.145505 i.e. < Y_{UB}

ii) For x = 0.30

Y_L = 2.77489 - 1.18921 = 1.58568 i.e. < Y_{UB}

iii) For x = 0.35

Y_L = 3.174305 - 1.1756195 = 1.998685 i.e. < Y_{UB}

iv) For x = 0.36

Y_L = 3.254188 - 1.1730033 = 2.081185 i.e. > Y_{UB}

X_{DL} = 0.36 µg Zn/ml

Table 102 : Fitted line data for Zn by FAAS with Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations y

X Concn. of Zn µg/ml	Y ILL 151 DIGITAL READOUT	$\hat{y}$	WORKING-HOTELLING	CONFIDENCE BOUNDS
0.25	2.3	2.38	2.91 or 1.84	3.85 or 0.90
0.50	4.3	4.37	4.81 or 3.94	5.72 or 3.02
0.75	6.4	6.37	6.86 or 6.01	7.63 or 5.11
1.00	8.7	8.37	8.70 or 8.03	9.59 or 7.14
1.50	12.1	12.36	12.79 or 11.93	13.71 or 11.01
2.00	16.4	16.36	17.00 or 15.71	17.97 or 14.74

Table 103 : Fitted line data for Cd by FAAS with Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations y

X Concn. of Cd µg/ml	Y ILL 151 DIGITAL READOUT	$\hat{y}$	WORKING-HOTELLING	CONFIDENCE BOUNDS
0.25	2.8	2.94	3.55 or 2.33	5.31 or 0.57
0.50	5.1	5.21	5.71 or 4.72	6.08 or 4.35
0.75	7.4	7.49	7.90 or 7.08	8.21 or 6.77
1.00	10.4	9.77	10.14 or 9.39	10.43 or 9.10
1.50	14.1	14.32	14.81 or 13.83	16.49 or 12.15
2.00	18.8	18.87	19.62 or 18.13	20.17 or 17.58

Table 104 : Fitted line data for Pb by FAAS with its Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations y

X Concn. of Pb µg/ml	Y ILL 151 DIGITAL READOUT	$\hat{y}$	WORKING-HOTELLING	CONFIDENCE BOUNDS
0.50	1.0	1.29	1.77 or 0.82	2.65 or -0.06
1.00	2.5	2.35	2.84 or 1.85	3.62 or 1.07
2.00	4.5	4.46	4.82 or 4.09	5.45 or 3.47
4.00	8.9	8.68	9.00 or 8.36	9.84 or 7.52
6.00	12.9	12.90	13.32 or 12.48	14.19 or 11.61
8.00	17.0	17.12	17.72 or 16.52	18.63 or 15.61

Table 105 : Fitted line data for Cu by FAAS with its Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations y

X Concn. of Cu µg/ml	Y ILL 151 DIGITAL READOUT	$\hat{y}$	WORKING-HOTELLING	CONFIDENCE BOUNDS
0.25	0.7	0.65	0.93 or 0.36	1.47 or -0.18
0.50	1.3	1.25	1.52 or 0.98	0.56 or 1.94
1.00	2.3	2.46	2.71 or 2.22	3.25 or 1.68
2.00	4.8	4.89	5.10 or 4.68	5.53 or 4.24
4.00	10.0	9.74	9.94 or 9.53	10.47 or 9.00
6.00	14.5	14.58	14.87 or 14.30	15.41 or 13.76
8.00	19.4	19.43	19.83 or 19.03	20.68 or 18.18

Table 106 : Fitted line data by DPASV-HMDE with Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations y

a) Zn

X Concn. of Zn ng/ml	Y PEAK HEIGHT (mm)	$\hat{y}$ (mm)	WORKING-HOTELLING	CONFIDENCE BOUNDS
0.0	36.0	35.10	42.57 or 27.63	57.51 or 12.69
20.0	71.0	71.70	76.59 or 66.81	90.42 or 52.98
40.0	107.0	108.30	113.19 or 103.41	127.02 or 89.58
60.0	146.0	144.90	152.37 or 137.43	167.31 or 122.49

b) Cd

X Concn. of Cd ng/ml	Y PEAK HEIGHT (mm)	$\hat{y}$ (mm)	WORKING-HOTELLING	CONFIDENCE BOUNDS
0.0	28.0	28.50	45.94 or 11.06	80.81 or -23.81
20.0	78.5	79.95	91.17 or 68.33	123.18 or 35.82
40.0	135.0	131.00	142.42 or 119.58	174.68 or 87.32
60.0	180.0	182.25	199.69 or 164.81	234.56 or 129.94

c) Pb

X Concn. of Pb ng/ml	Y PEAK HEIGHT (mm)	$\hat{y}$ (mm)	WORKING-HOTELLING	CONFIDENCE BOUNDS
0.0	17.5	17.55	19.08 or 16.02	22.13 or 12.97
20.0	39.0	39.10	40.10 or 38.10	43.54 or 34.66
40.0	61.0	60.65	61.65 or 59.65	65.09 or 56.21
60.0	82.0	82.20	83.72 or 80.67	86.78 or 77.62

d) Cu

X Concn. of Cu ng/ml	Y PEAK HEIGHT (mm)	$\hat{y}$ (mm)	WORKING-HOTELLING	CONFIDENCE BOUNDS
0.0	33.0	31.5	41.49 or 21.51	61.45 or 1.55
20.0	62.0	64.0	70.54 or 57.46	89.01 or 38.99
40.0	96.0	96.5	108.04 or 89.96	121.51 or 71.49
60.0	130.0	129.0	138.99 or 119.01	158.95 or 99.05

Table 107 : Fitted line data by DPASV-RDE with Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations y

a) Cd

X Conc. of Cd ng/ml	Y PEAK HEIGHT (mm)	$\hat{y}$ (mm)	WORKING-HOTELLING	CONFIDENCE BOUNDS
0.00	4.5	4.56	6.00 or 3.12	8.88 or 0.24
13.33	8.5	8.57	9.51 or 7.63	11.38 or 5.76
26.67	12.9	12.58	13.52 or 11.64	15.39 or 9.77
40.00	16.4	16.59	18.03 or 15.15	20.91 or 12.27

b) Pb

X Concn. of Pb ng/ml	Y PEAK HEIGHT (mm)	$\hat{y}$ (mm)	WORKING-HOTELLING	CONFIDENCE BOUNDS
0.00	6.5	6.49	7.74 or 5.24	10.23 or 2.75
13.33	12.6	12.48	13.29 or 11.66	15.16 or 9.80
26.67	18.2	18.47	19.29 or 17.66	21.15 or 15.79
40.00	24.6	24.46	25.71 or 23.21	28.20 or 20.72

c) Cu

X Concn. of Cu	Y PEAK HEIGHT (mm)	$\hat{y}$ (mm)	WORKING-HOTELLING	CONFIDENCE BOUNDS
0.00	5.0	5.00	5.20 or 4.80	5.50 or 4.40
13.33	8.5	8.57	8.63 or 8.37	9.00 or 8.00
26.67	12.0	12.00	12.13 or 11.87	12.50 or 11.50
40.00	15.5	15.50	15.70 or 15.30	14.90 or 16.10

Table 108 : Fitted line data by GFAAS with Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations y

X Concn. of Pb ng/ml	Y PEAK HEIGHT (mm)	$\hat{y}$ (mm)	WORKING-HOTELLING	CONFIDENCE BOUNDS
0	0.00	0.016	0.11 or -0.08	0.27 or -0.24
2	0.30	0.270	0.34 or 0.20	0.49 or 0.05
4	0.50	0.524	0.58 or 0.47	0.73 or 0.32
6	0.80	0.778	0.85 or 0.71	1.00 or 0.56
8	1.02	1.032	1.13 or 0.94	1.29 or 0.77

Determination of detection limit for method using standard additions

Detection limit at the 99% confidence limit is given by :-

$$\Delta \bar{X}_{lim} > t.\ S_{xb} \sqrt{\frac{1}{n} + \frac{1}{n}}$$

where,

$\Delta \bar{X}_{lim}$ = detection limit

t = students t value at the 95% confidence limit and n-2 degrees of freedom

S_{xb} = standard deviation of the blank values

n = number of observations.

Thus for Zn at the HMDE,

$$\Delta \bar{X}_{lim} > 7.173 \times 0.81650 \sqrt{\frac{1}{6} + \frac{1}{6}} = \underline{3.6 \text{ ng/ml}}$$

Figure 92 : Fitted line for Cd by FAAS with Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations

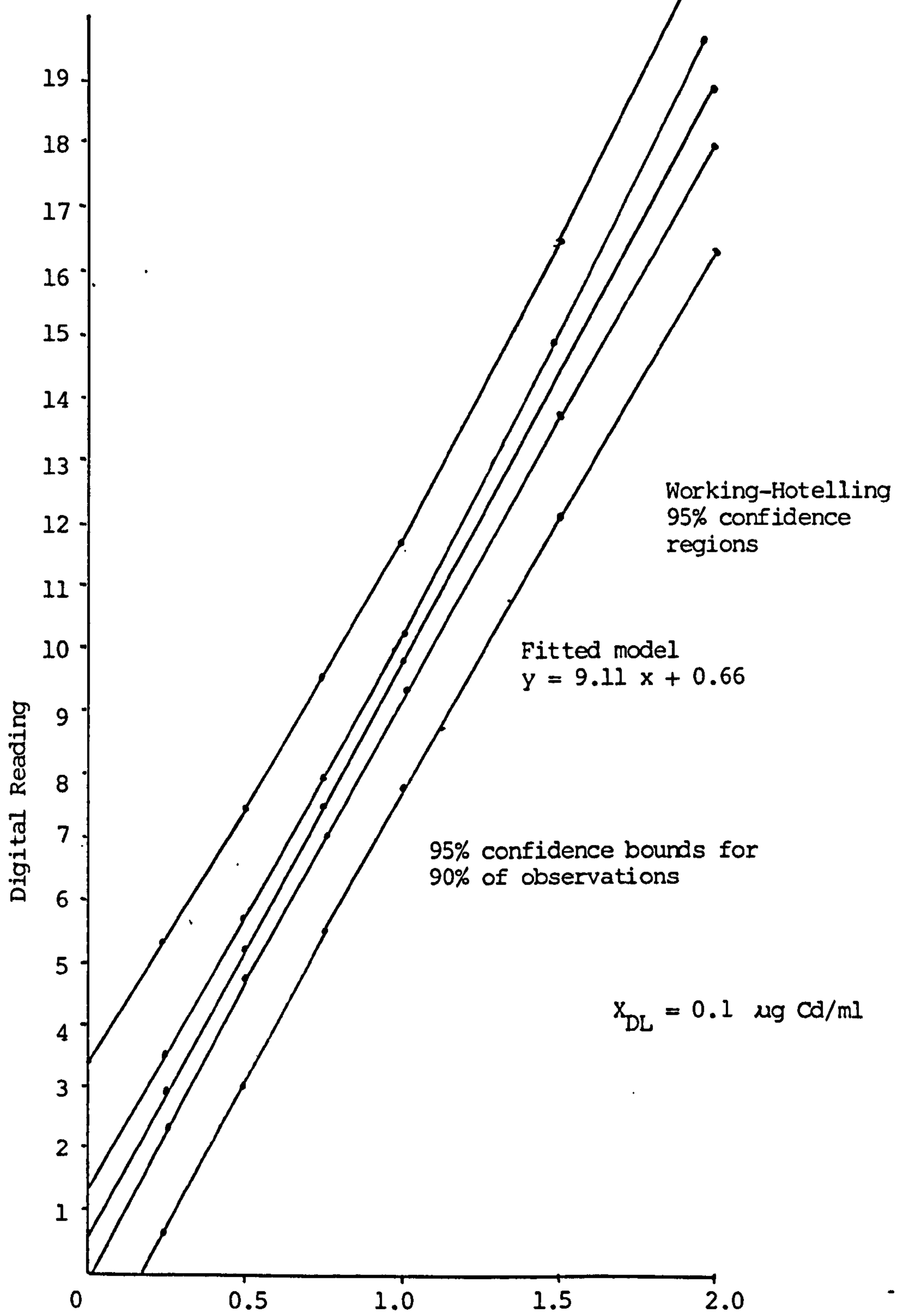


Figure 93 : Fitted line for Pb by FAAS with its Working-Hotelling 95% confidence regions plus the confidence bounds for 90% of future observations

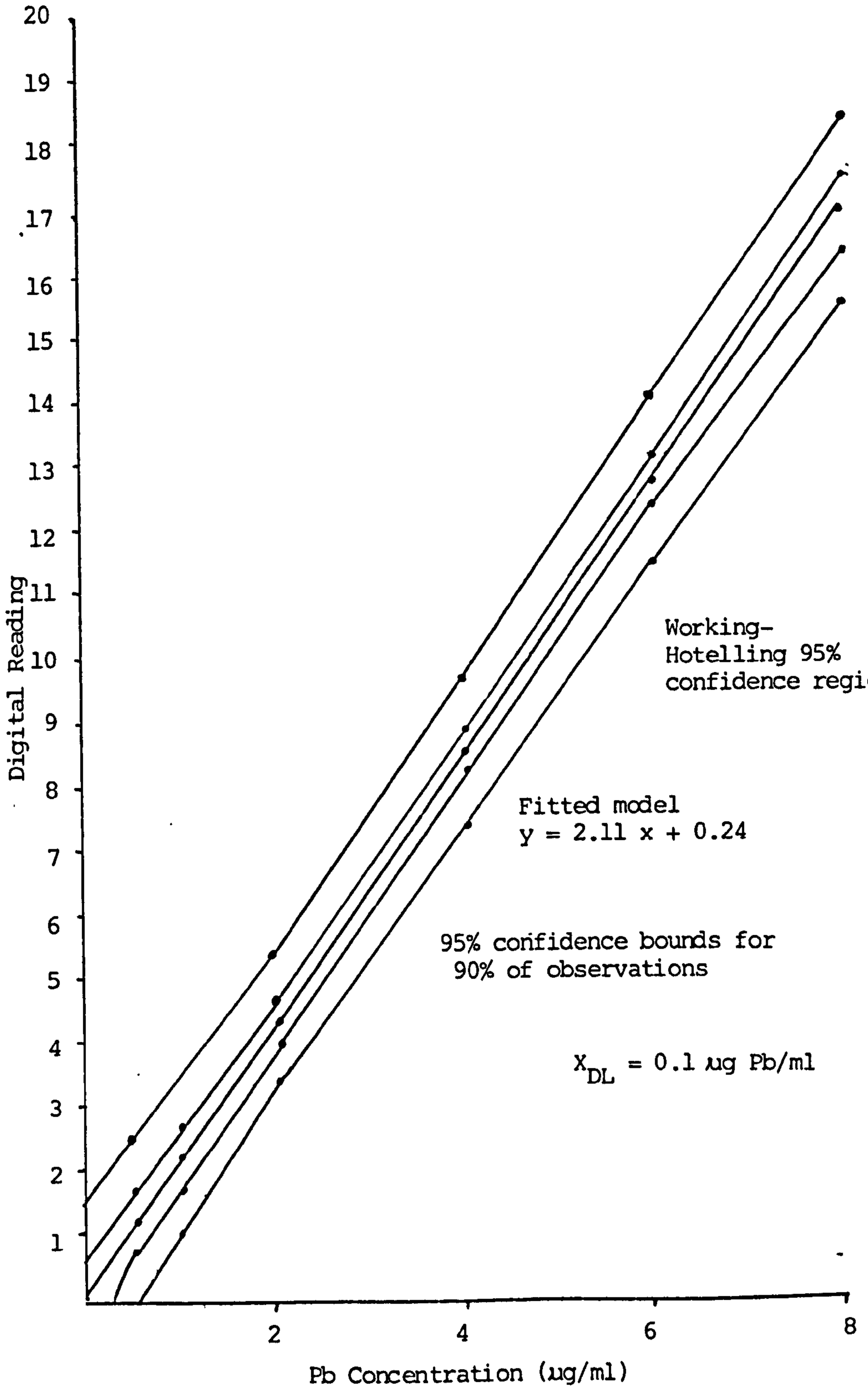


Figure 94 : Fitted line for Cu by FAAS with its Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations

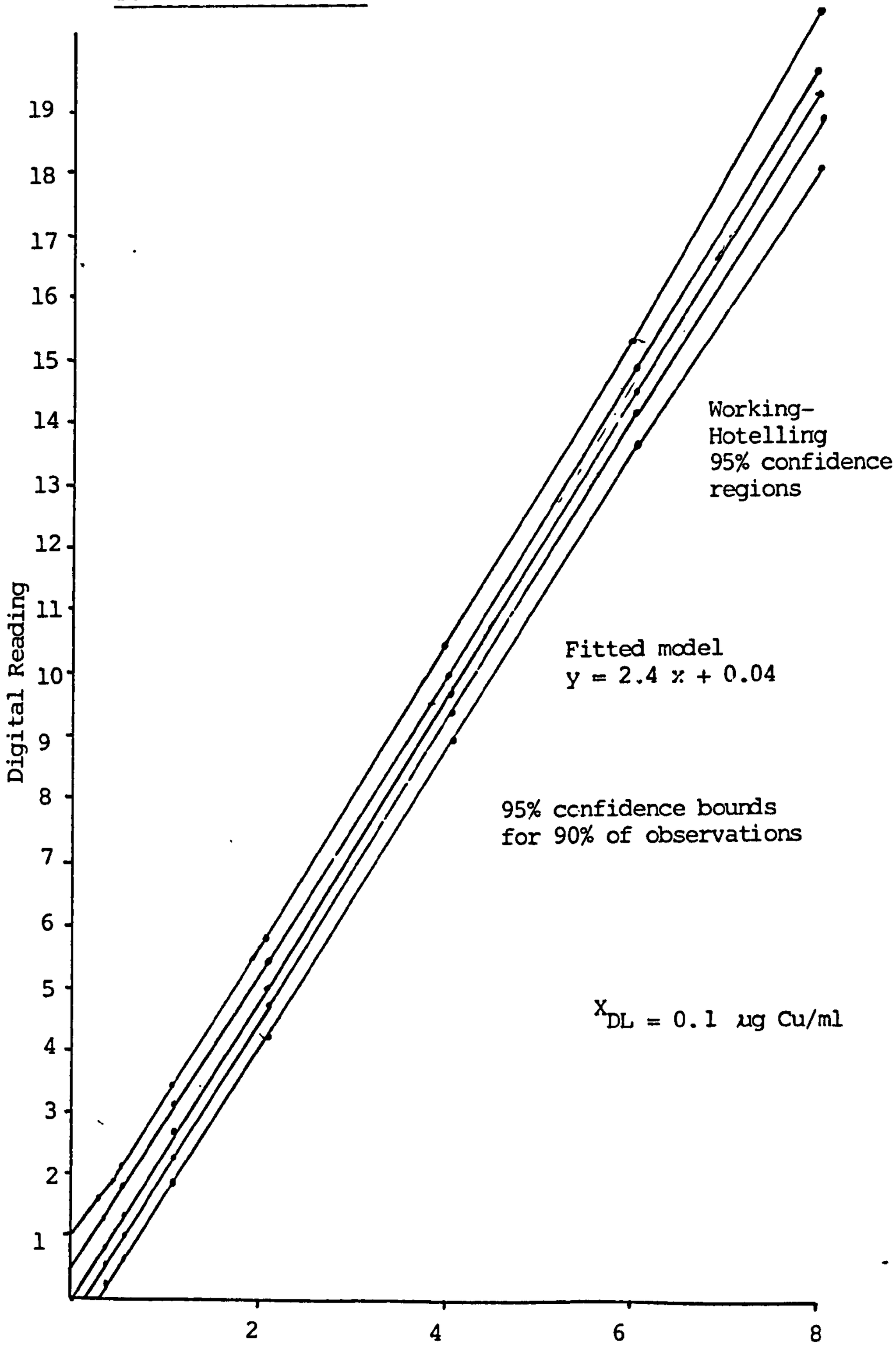
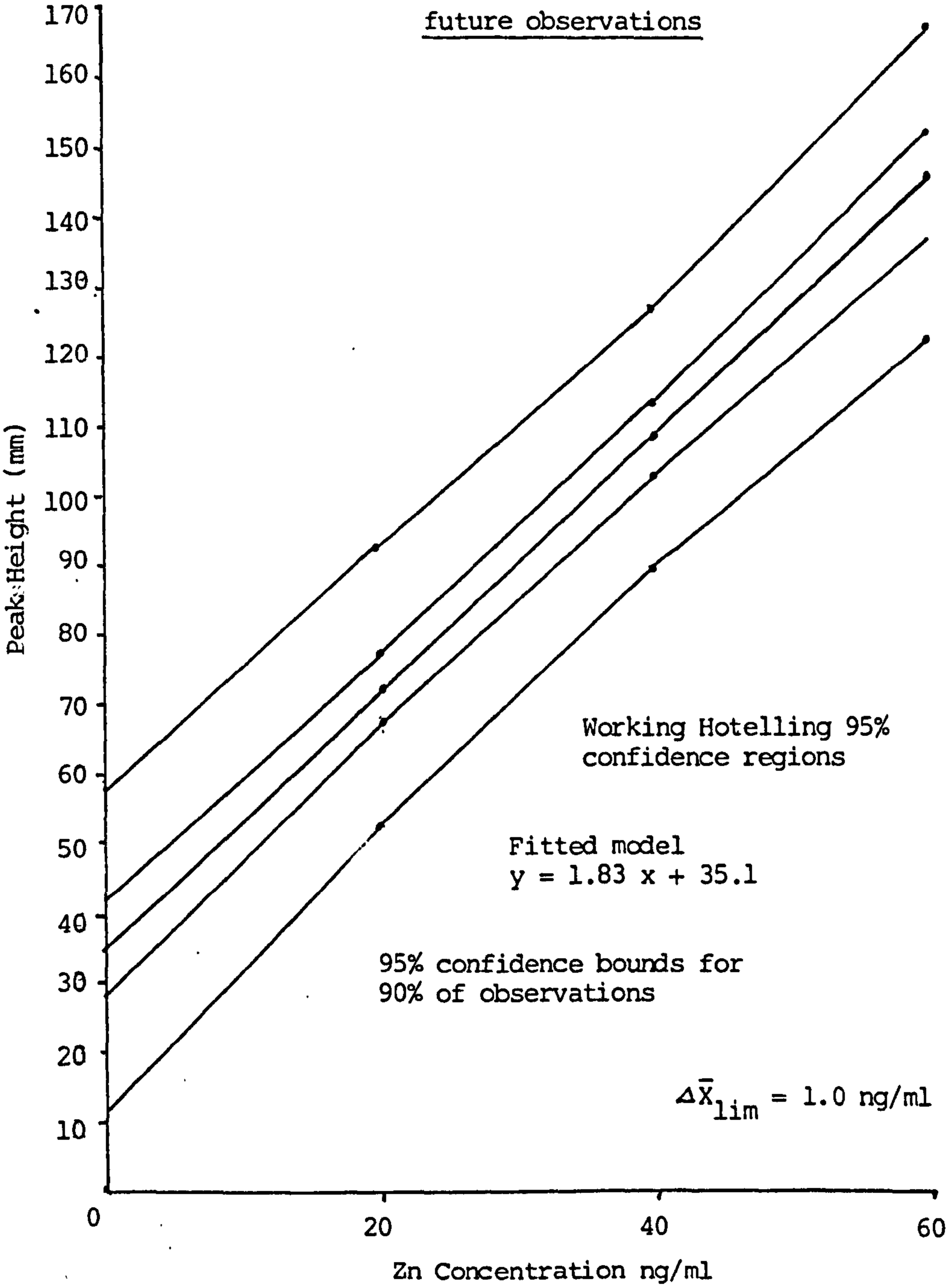


Figure 95 : Fitted line for Zn by HMDE with its Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations



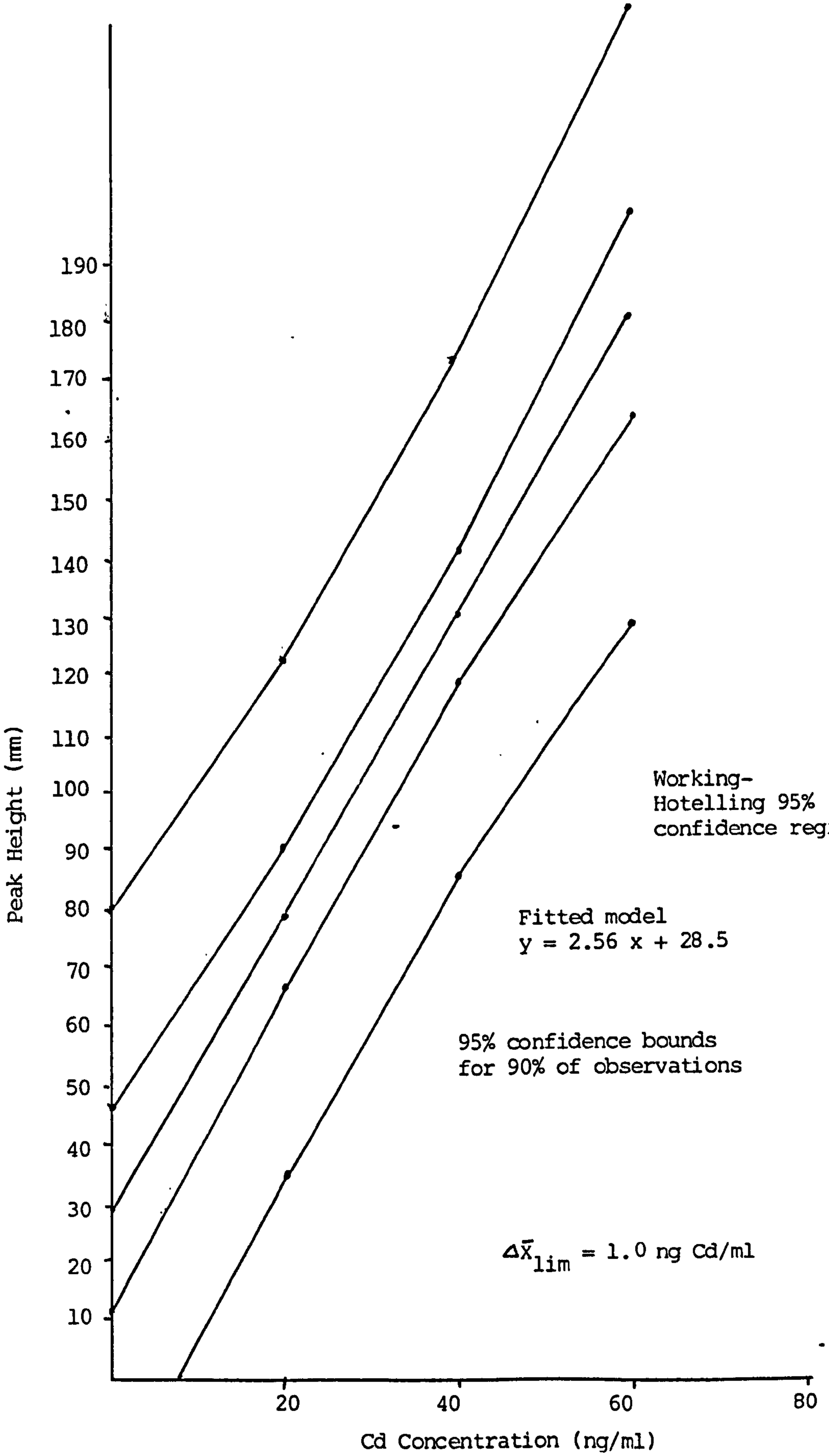


Figure 96 : Fitted line for Cd by HMDE with its Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations

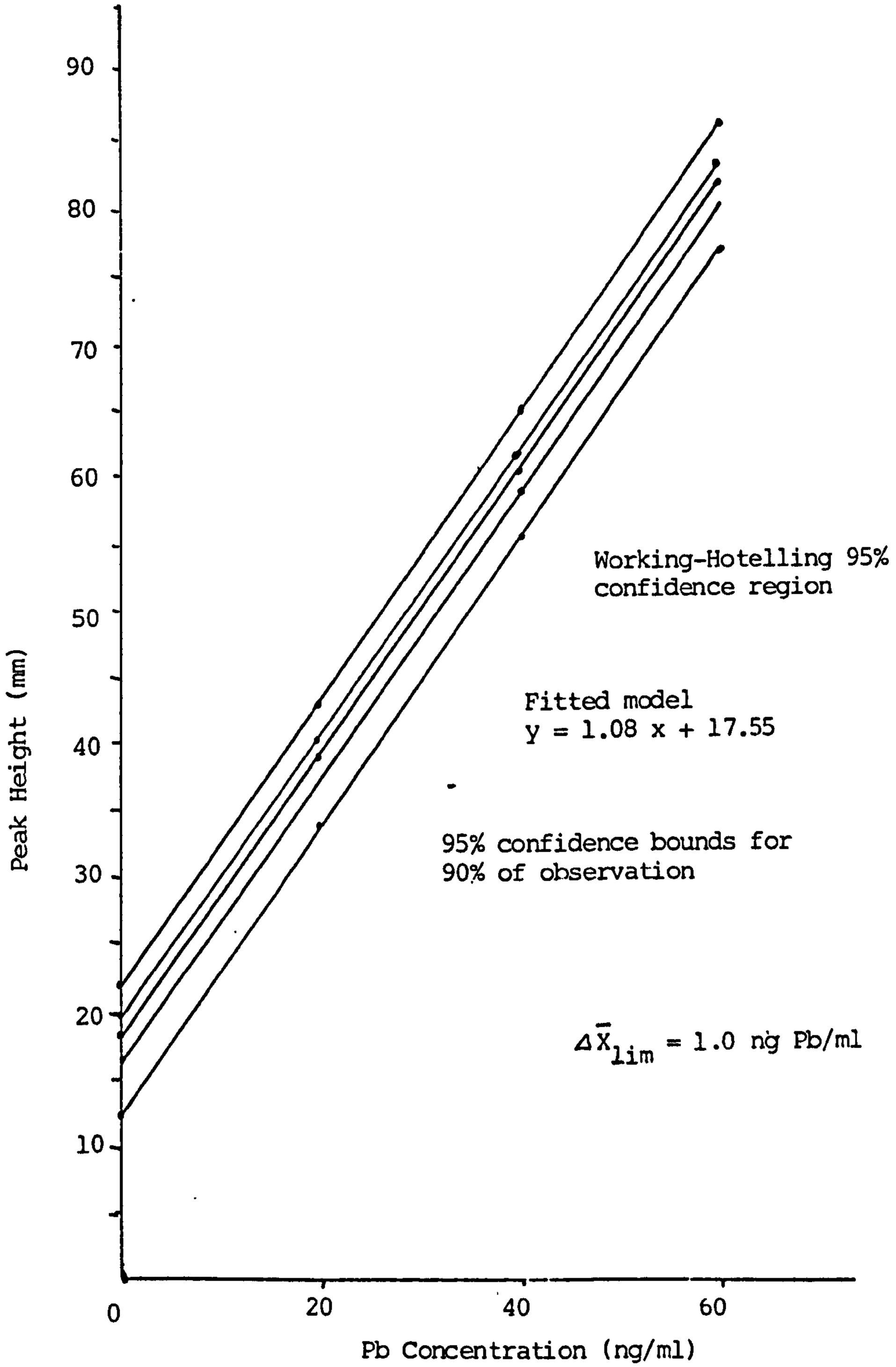


Figure 97 : Fitted line for Pb by HMDE with its Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations

Figure 98 : Fitted line for Cu by HMDE with its Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations

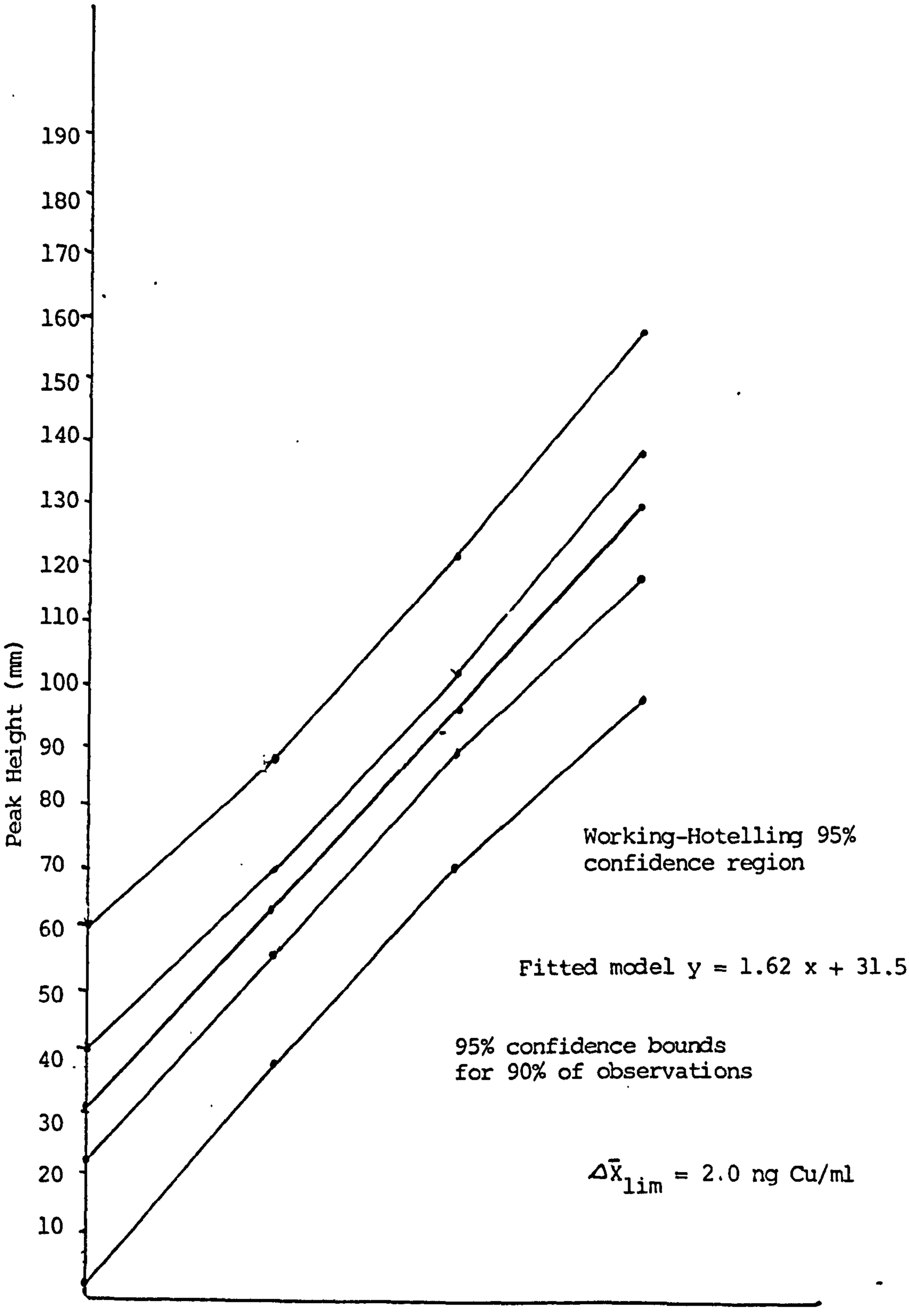
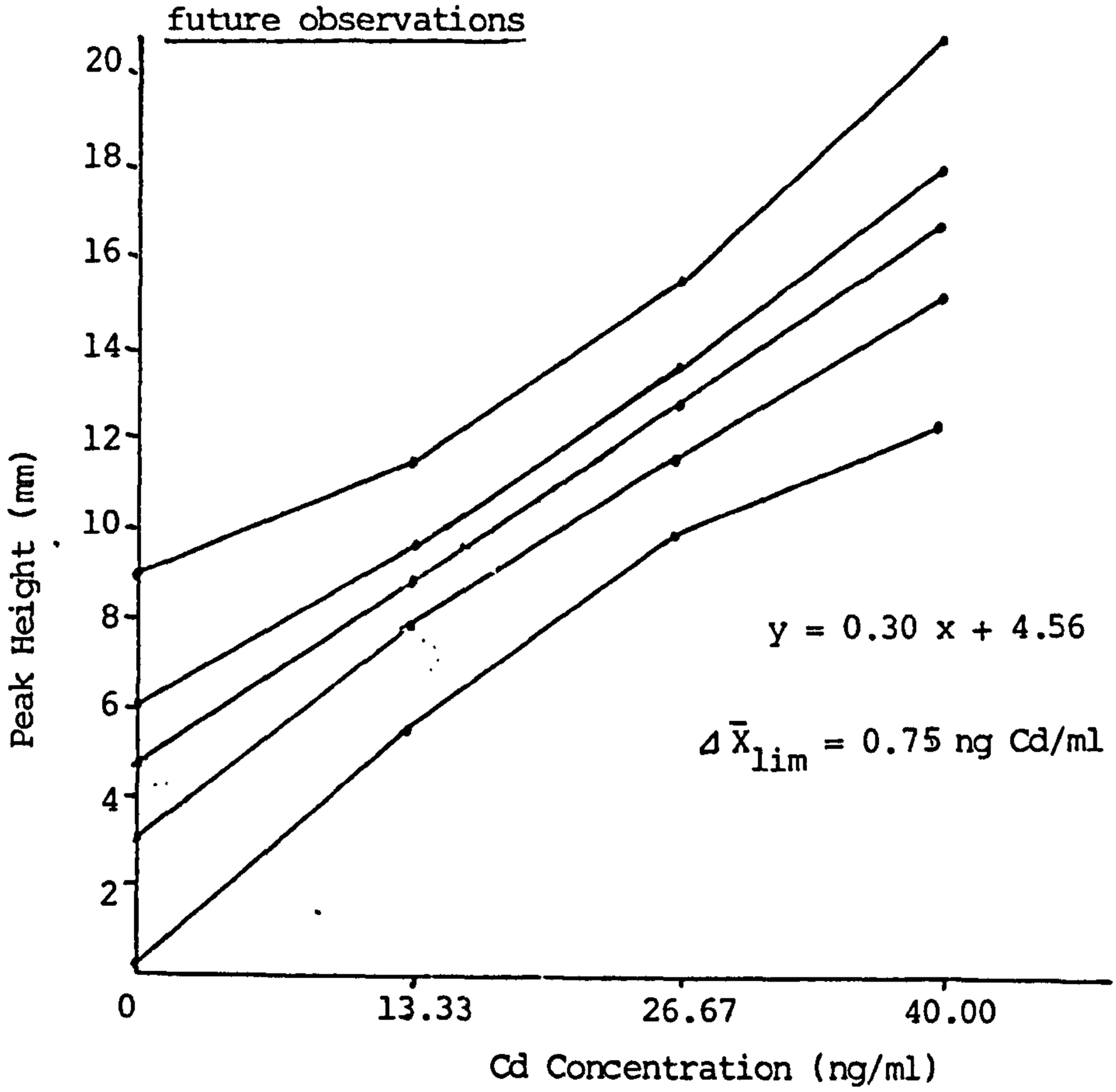


Figure 99 : Fitted lines for a) Cd and b) Cu by RDE with Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations

a) Cd



b) Cu

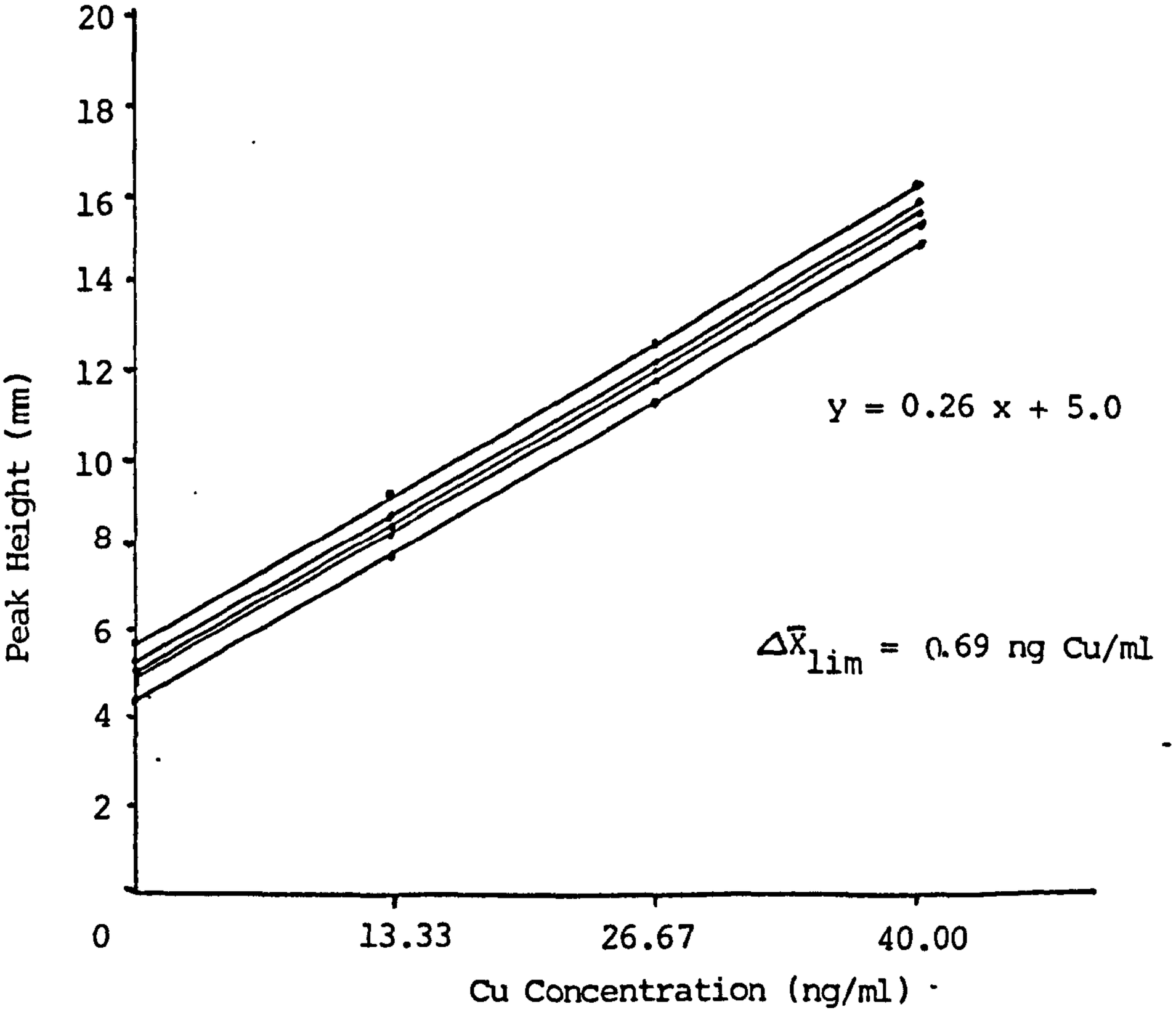


Figure 100 : Fitted lines for Pb by RDE with Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations

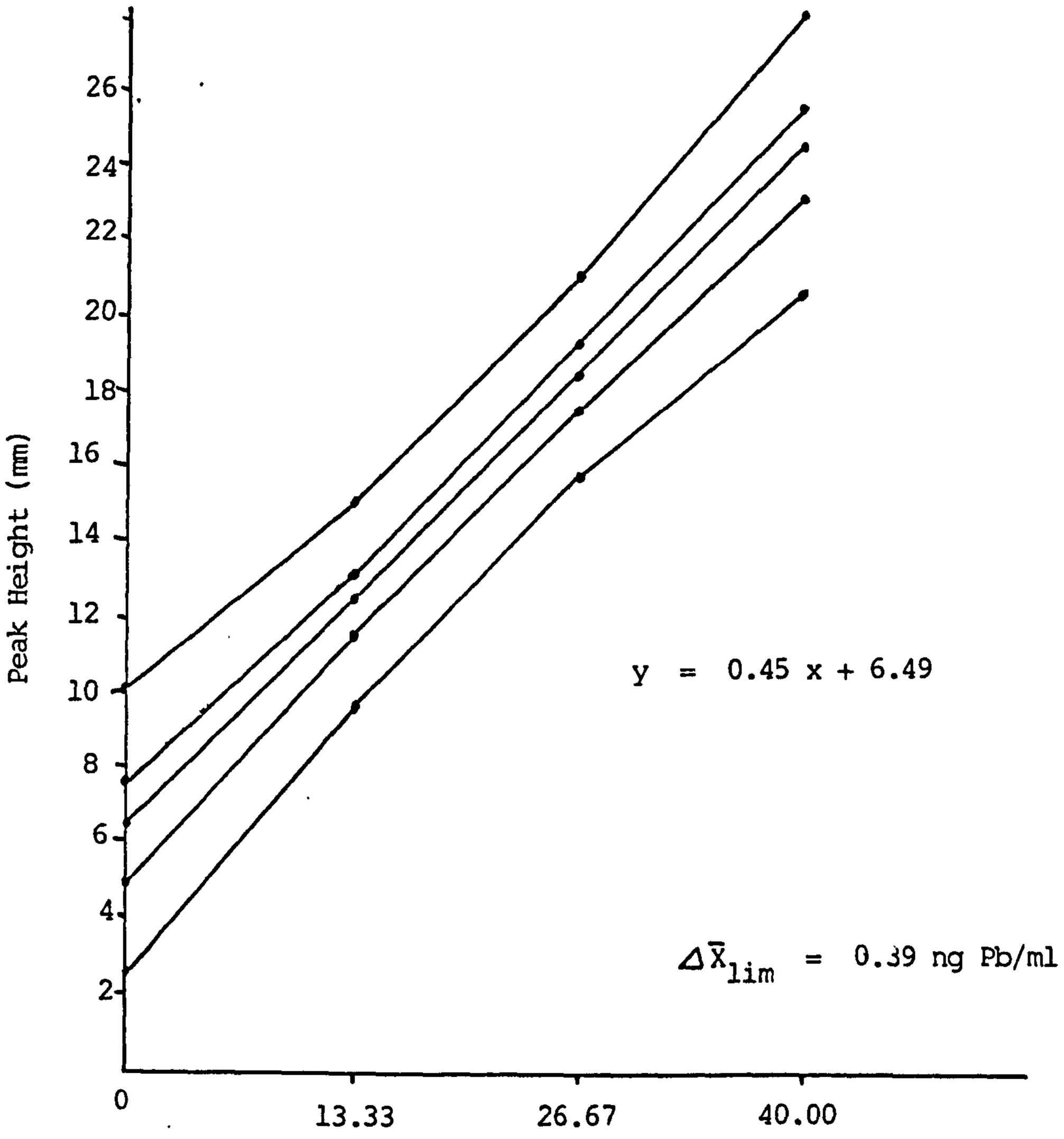


Figure 101 : Fitted line for Pb by GFAAS with Working-Hotelling 95% confidence region plus the confidence bounds for 90% of future observations

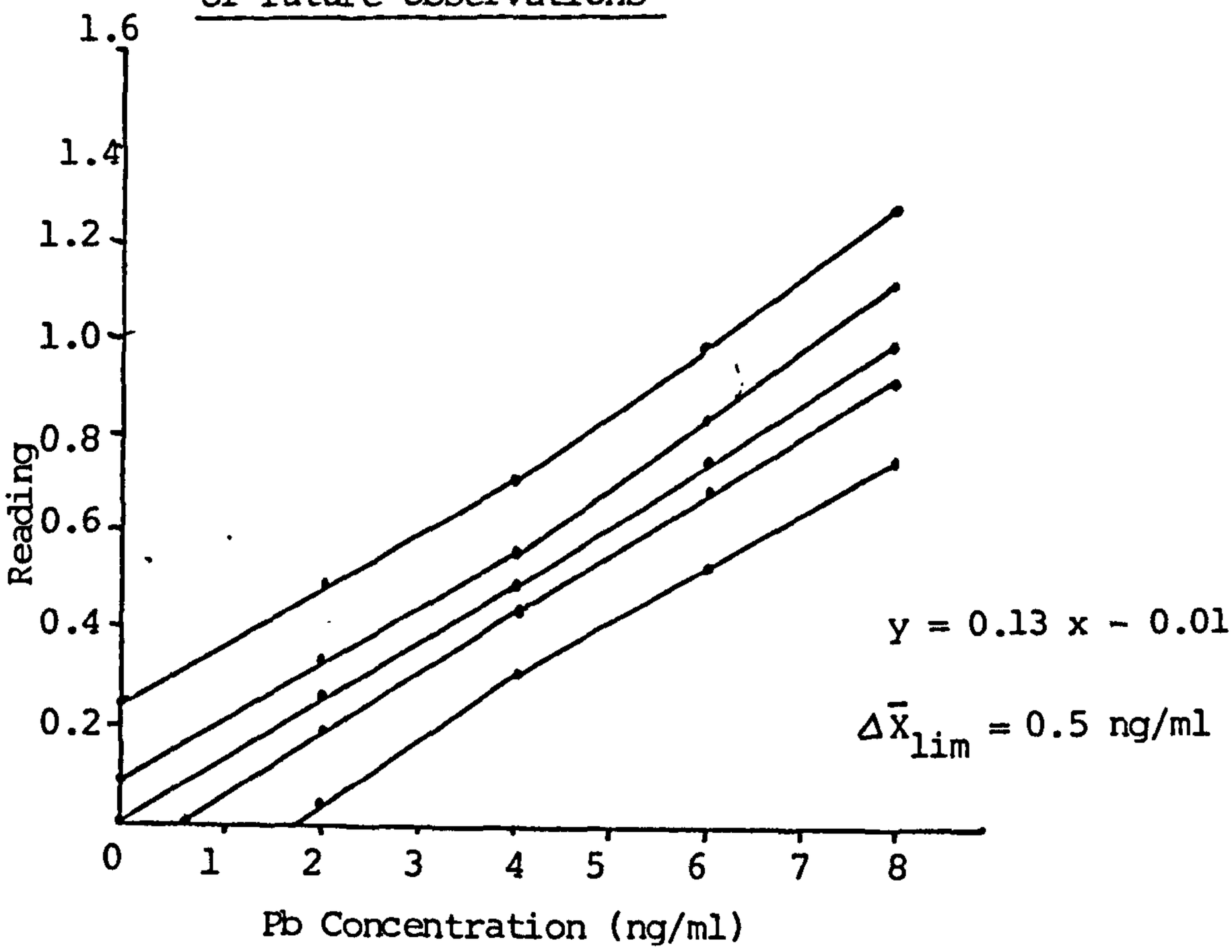


Figure 102 : Cd line by GFAAS

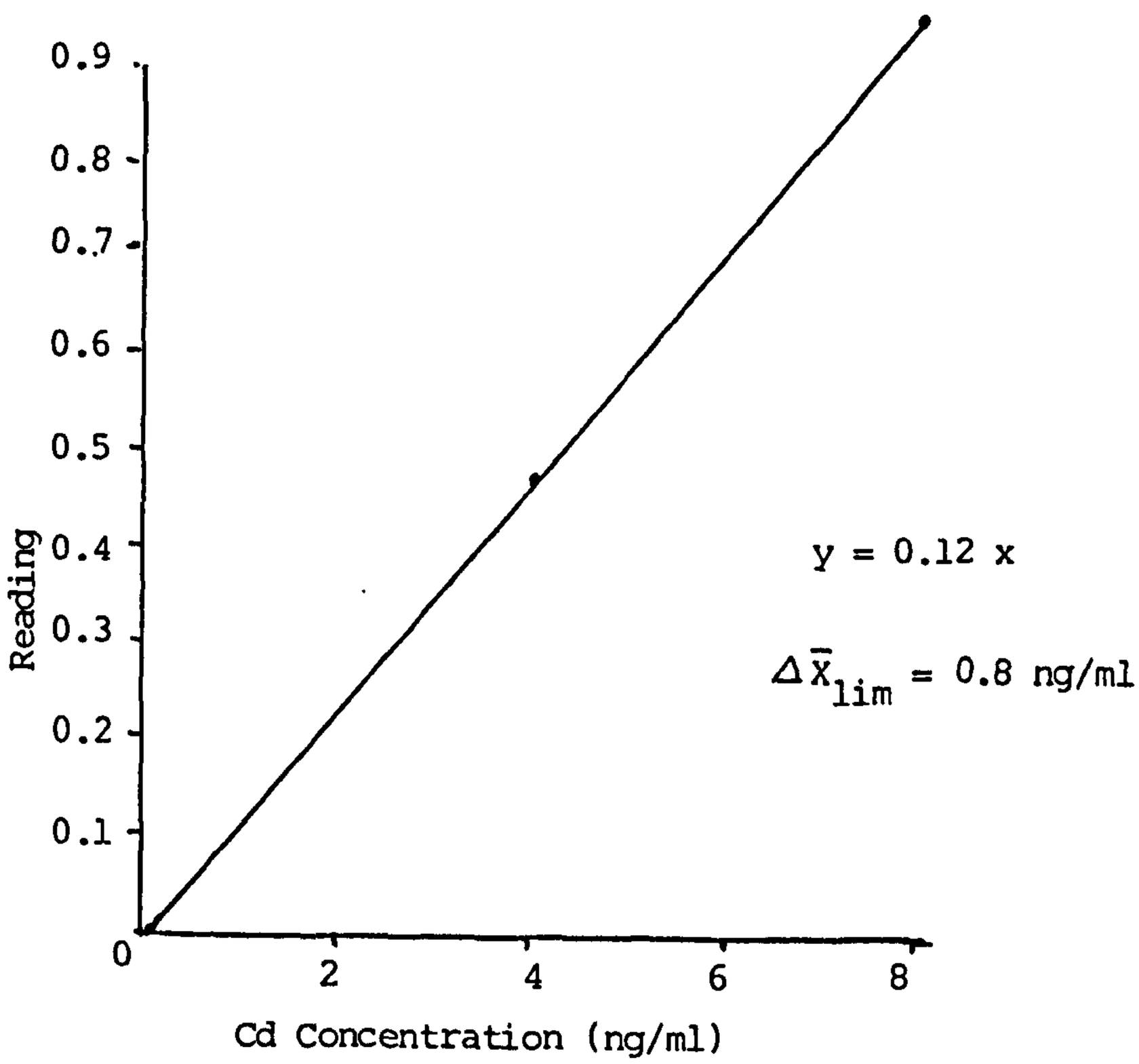
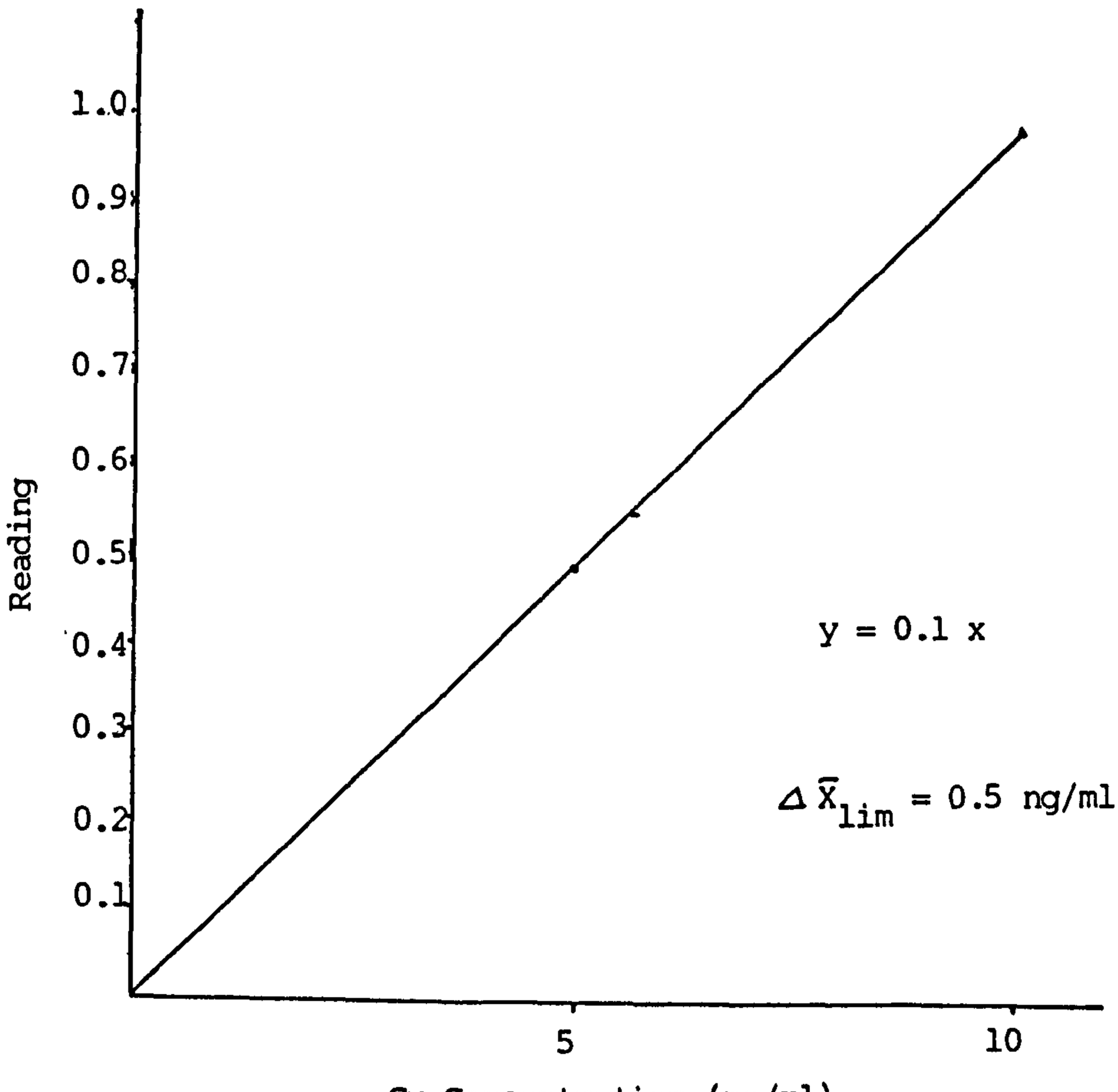


Figure 103 : Cu line by GFAAS



SECTION 8 : INVESTIGATION OF SOURCES OF CONTAMINATION

8.1 Experimental Preparation

a) Reagents

b) Preparation of Standard Solutions

c) Sample Description

d) Sample Treatment, Analysis and Results

e) Apparatus and Instrumental Parameters

SECTION 8 : INVESTIGATION OF SOURCES OF CONTAMINATION

a) Reagents

AristaR nitric acid.

AnalaR cadmium nitrate, copper, nitrate, lead nitrate and zinc nitrate.

b) Preparation of standard metal solutions for GFAAS

Calibration standards were prepared from the stock metal solution by spiking a matched nitric acid solution with aliquots of 100 µg/ml metal solution (prepared from the stock metal solution) using a micropipette.

Four calibration standards were used for the Pb determinations and two for each of the Cd and Cu analyses.

c) Sample description

The samples consisted of :-

1) Blue towel paper - a section from the centre of 3 consecutive towels.
2) Mediwipe tissues.
3) Whatman 541 filter paper (not handled).
4) Whatman 541 filter paper (handled).
5) Whatman membrane filters 5, 0,45 and 0.1 µm (pore size).
6) Separator papers from between the filters 5, 0.45 and 0.1 µm (pore size).

Such materials are frequently used during analytical procedures.

d) Sample treatment, analysis and results

The samples were acid digested twice with concentrated nitric acid.

Analysis for Cd, Cu and Pb was carried out by GFAAS, and for Zn by FAAS.

The results obtained are given in Table 109.

Table 109: Metal concentrations in materials used during the analytical process

	Concentration ng/g*			
Sample	Zn*	Cd	Pb	Cu
Mediwipe 1	8.0	N.D.	N.D.	N.D.
Mediwipe 2	2.8	N.D.	8.6	2.2
Mediwipe 3	2.2	N.D.	N.D.	1.9
Mean	4.3	-	2.9	1.4
Blue towel 1	21.0	3.0	219.3	5.0
Blue towel 2	24.8	1.3	118.7	N.D.
Blue towel 3	56.2	N.D.	N.D.	N.D.
Mean	34.0	1.4	112.7	1.7
Whatman 541 1	1.8	2.0	12.1	N.D.
Whatman 541 2	0.3	N.D.	N.D.	2.2
Mean	1.1	1.0	6.1	1.1
Handled Whatman 541	2.8	N.D.	N.D.	2.4
Separator (5 µm) 1	N.D.	N.D.	1.4	N.D.
Separator (5 µm) 2	14.6	N.D.	1.8	N.D.
Separator (5 µm) 3	20.8	N.D.	0.9	27.8
Mean	11.8	-	1.4	9.3

* Zn analysis by FAAS, results in µg/g

Table 109 continued.

Sample	Concentration ng/g*			
	Zn*	Cd	Pb	Cu
5 µm filter 1	N.D.	N.D.	N.D.	27.8
5 µm filter 2	0.33	N.D.	N.D.	N.D.
5 µm filter 3	N.D.	N.D.	N.D.	N.D.
5 µm filter 4	3.3	N.D.	N.D.	7.9
Mean	0.9	-	-	8.9
Separator (0.45) 1	N.D.	N.D.	N.D.	7.9
Separator (0.45) 2	N.D.	N.D.	N.D.	N.D.
Mean	-	-	-	4.0
0.45 filter 1	6.5	2.5	1.2	N.D.
0.45 filter 2	10.3	N.D.	N.D.	N.D.
0.45 filter 3	2.4	1.9	N.D.	N.D.
0.45 filter 4	N.D.	N.D.	N.D.	N.D.
Mean	4.8	1.1	0.3	-
0.1 Separator 1	8.1	N.D.	4.8	13.9
0.1 Separator 2	5.8	5.3	N.D.	N.D.
Mean	7.0	2.7	2.4	7,0
0.1 filter 1	10.6	N.D.	N.D.	N.D.
0.1 filter 2	N.D.	N.D.	N.D.	N.D.
0.1 filter 3	14.8	N.D.	N.D.	N.D.
0.1 filter 4	3.2	N.D.	N.D.	N.D.
Mean	7.2	-	-	-

* Zn analysis by FAAS, results in µg/g.

e) Apparatus and instrumental parameters

FAAS

See Part II, Section 1.

Analysis by GFAAS of heavy metals in prepared sample solution

Apparatus :-

Pye Unicam SP-9 atomic absorption spectrophotometer, equipped with a Massman graphite cuvette

Pye Unicam SP-9 computer

Pye Unicam SP-9 video furnace programmer

Pye Unicam SP-9 autoanalyser.

Instrumental parameters

Spectrophotometer parameters :-

	Wavelength (nm)	Lamp Current (mA)	Band pass (nm)
Cd	228.8	7	0.5
Cu	324.8	7	0.5
Pb	217.0	5	0.5

Electrothermal Heating Process

	Phase	Process	Temperature (°C)	Time (s)	Ramp (°C/s)
Cd	1	Drying	135	30	100
	2	Ashing	350	15	50
	3	Atomisation	1150	2	> 2000
	4	-	0	5	> 2000
	5	Cleaning	2300	2	> 2000
Cu	1	Drying	120	30	100
	2	Ashing	1050	15	50
	3	Atomisaticn	2650	8	> 2000
	4	-	0	5	> 2000
	5	Cleaning	2900	2	> 2000
Pb	1	Drying	125	20	50
	2	Ashing	500	40	200
	3	Atomisation	1600	3	> 2000
	4	Cleaning	2500	2	> 2000

PART III

DISCUSSION

DISCUSSION

a) Purification Methods

Despite the increasing number of analytical techniques with detection limits spanning the sub-µg/g through the sub-ng/g range; analyses of real samples for ultratrace elements (< 0.1 µg/g) are not performed routinely with a relative error of < ± 10%. High sensitivity alone is an insufficient condition for obtaining accuracy and precision - sensitivity limits of analytical methods lie well below the level at which chemists are able to make accurate determinations (273). One of the most restricting parameters is the problem of control of contamination. In the present work, a number of techniques were applied in an attempt to control the level of blank contamination. Of these, the method of isothermal distillation was found to provide a simple and economical means of producing high purity acids. For the more volatile acids, such as acetic acid for example,the process was fairly rapid, producing an 8 M solution within one week. For the less volatile nitric acid the process took much longer. The rate of distillation need not be a problem, since the molarity of the distilled acid may be increased either by renewing the reagent grade acid, or the number of acid containers.

The method is reliant on the purity of the water used, and is therefore limited by the efficiency of the water purification methods. Tap water collected in Bristol University was found to be contaminated with zinc, cadmium, lead and copper (Table 27). Copper, in particular, was a problem (360 ng/ml). Dilution of the tap water with distilled water was necessary for quantitative analysis by DPASV. Deionised and single distilled water were also found to be contaminated (Figures 18 and 20). Deionisation improved the Cd and Pb levels (2.0 and 4.0 ng/ml respectively compared with 40 ng/ml Cd and 120 ng/ml

Pb in tap water). The Zn and Cu levels were lower than those of tap water, but still too high for quantitative analysis without dilution. The Zn and Cu concentrations of DDW (22.0 and 4.0 ng/ml respectively) were much lower than those of deionised water (116 and 154 ng/ml), while the concentrations for Cd (24 ng/ml) and Pb (6.0 ng/ml) were higher (i.e. 2.0 and 4.0 ng/ml respectively for DI water). After passage through the Amberlite IRC-718, the metal levels in DDW were greatly decreased (i.e. 1.0, 1.0 and 4.0 ng/ml for Zn, Cd and Pb respectively. Cu was not detected; Tables 28 and 29 and Figure 18). The method is simple, economical and rapid for small volumes. Mitchell (273) suggested ion exchange as a means of water purification, and other authors (177) have recommended Amberlite IRC-718 for purification of background electrolytes. However, the resin's efficiency decreases below pH 4. Thus for the purification of the pH 3 buffer, 0.2 M ammonium citrate, it was found necessary to pass the electrolyte through the column at a pH of 4, and then add sufficient citric acid to bring the pH to 3. After passage through the column none of the four metals was detected in any of the buffers.

Figure 19 indicates the level of metal contamination found in water collected from a Millipore "Milli-Q" water system; distillation of the water was necessary to produce the required purity.

Double distillation of tap water in an all glass still was found to produce water of sufficient purity and quantity. Triple distillation produced very pure water (Figure 18), but was time consuming, expensive, and was used only for very dilute samples.

Purification of the background electrolytes by solvent extraction proved to be very time consuming and not as economical as Amberlite IRC-718. Figures 21 and 22 indicate that contamination remained a problem after solvent extraction. Exposure to other reagents, glassware and the

atmosphere during the multiple steps required for solvent extraction led to further contamination of the electrolyte.

Mercury cathode electrolysis provided a simple and rapid means of purifying electrolytes. Large volumes could be processed and transferred directly to the polarographic cell. The equipment is available commercially (293) or can be easily constructed (Figure 23). Table 30 and Figure 24 indicate the degree of purity attainable by the method.

b) Water sample analysis

Rheidol and Ystwyth water samples

The Rheidol samples were found to be contaminated with Zn, Cd, Pb and Cu (Table 31) as would be expected from the long history of base metal mining in the area.

Table 31 indicates an increasing zinc concentration as the Cwm Rheidol adit passes over mining waste. The Cd, Pb and Cu levels were much lower than that of Zn. In general, the Cd, Pb and Cu levels increased as the adit progressed downwards, across the mine dumps.

The Ystwyth samples also show contamination with Zn, Cd, Pb and Cu, again not surprising considering the area's association with Pb-Zn mining. The Zn concentration, in particular, was high, necessitating the use of FAAS rather than DPASV as the means of quantitative analysis, as was found for the Rheidol samples. The dilution effect of the river on the adit is demonstrated in Table 31.

From the results given in Table 31, it is easy to see how metal contamination can be carried downstream, leading to the pollution of the floodplain and eventually Cardigan Bay.

Table 32 indicates that, as a general rule, the Zn concentration of the Ystwyth and Rheidol samples, Y_1 and R_5, decreased with the decrease of the size fractions. In general, a large proportion of the Zn was

associated with particles whose dimensions were between 0.1 and 5 μm. The deviation from this rule, shown by $Y_1 < 0.45$ μm and $Y_1 < 0.1$ μm may be due to sample contamination with Zn or organic material leached from the filter or blockage of the filter. (The samples were buffered at pH 3, 0.2 M ammonium citrate, as it was hoped to carry out this analysis by DPASV - pH 3 is claimed to release all of the four metals considered, except organically bound metal (294). However, in order to avoid dilution steps at this point, FAAS was used.)

In general, the Zn concentration of sample Y_3 (Figure 26) increased as the pH decreased. The natural pH of this sample was 6.9. It is reasonable to assume that heavy metals may be found as :-

1. Labile metals at pH 7.0
2. Labile metals plus metals desorbed from colloids at pH 5.0.
3. Labile metals plus metals desorbed from colloids plus metals associated with metal-organic complexes at pH 3.0. Thus, the lower the pH the higher the metal concentration.

The results in Table 33 indicate that Zn was found as labile metal as well as "bound" metal. The acid digest procedure produced the highest Zn concentration, probably due to the release of adsorbed Zn from dissolved organic matter, colloidal and mineral material which requires a more destructive treatment such as an acid attack. From the difference in Zn levels at pH 3 and for the acid digest, the majority of the Zn present in the sample was associated with the dissolved organic matter, colloidal and mineral material.

The Y_3 pH 5 determination was repeated. A low value was again obtained. The problem was encountered later (Table 34) for sample Y_4 and was alleviated to some extent by the use of an ammonium citrate pH 5 buffer. A matrix interaction - possibly with organic matter - may take place, causing Zn adsorption and hence depletion of the Zn peak. The

problem was not encountered with the Rheidol R_1 sample.

Table 34 shows that as a general rule the Zn concentration increases with decreasing pH and decreases with decreasing size fraction. The Zn in samples R_1 and Y_4 was present in both labile and "bound" forms, including organic, colloidal and mineral adsorption. The Zn in the Rheidol sample was at a high level in the particulate form - possibly as zinc blende, the mineral previously mined at this site. The release of Zn at pH 3 indicates Zn was also associated with organic complexes.

The Zn in the Ystwyth sample was found to be a predominantly labile form, with a smaller proportion associated with organic matter or colloidal and mineral material. The metal existed mainly in a form which passed through both the 0.45 and 0.1 μm filters.

Table 35 indicates the effectiveness of the limestone trap installed to control major contamination of the river by the adit drainage. High levels of Zn, Cd, Pb and Cu were found in the sample collected before passage through the trap. In particular Zn is at a high level - over three times the concentration considered to be toxic to plants grown in water culture (295). The metal levels after passage through the trap are substantially reduced, the metals being deposited as the carbonates.

The carbonate trap is, therefore, an effective means of pollution control. However, the limestone trap must be renewed periodically if it is to maintain its effectiveness and it is at these times that a "plug" of heavy metal pollution will pass into the river, which can further devastate plant and animal life downstream.

Caradon water sample

The Caradon water sample (Figure 27) had been acidified with nitric acid and was therefore not suitable for fractionation. The "total" Zn, Cd, Pb and Cu levels are given in Table 36. The sample was contaminated with Zn and Cu - particularly Cu, as was expected from the history of Cu mining in the area. It is apparent that although mining operations at South Caradon ended nearly a century ago, the remaining waste still leaches copper.

River Avon Samples

Table 37 indicates that the levels of Zn, Cd, Pb and Cu were not detectable or were present in low concentrations. The samples were collected on a day of heavy rainfall; the low metal levels could be due to a dilution effect. Higher metal levels were expected, especially at St. Annes (near the City centre) and Middlehill (near a sewage works). The results demonstrate the importance of weather conditions on pollution levels and also the importance of instrumental sensitivity and of low blank values.

Zn was found to be present at the highest concentration in both the Reybridge and Middlehill samples, but the Zn concentration was well below the median concentration of 15 µg Zn/l for fresh water (8). The Cd and Pb in the Reybridge sample were present in a labile form, while much of the Zn was "bound". In view of the clay nature of the river channel, Zn adsorbed onto clay particles is likely.

The Cd and Pb determined in the Middlehill sample was present in both labile and bound forms. Zn was present in a labile form. Interference at pH 3 prevented quantitation of the Zn level. But it can be conjectured that much of the Zn was present as the bound metal, since organic matter and clay material are likely to be present at this site. The interference

encountered at this pH may have been due to :-

i) the reduction of H^+ ions at -1.2 V vs. SCE, or

ii) the presence of an organic contaminant - the latter could explain the absence of Cu at this pH also.

Rainwater samples

Zn, Cd, Pb and Cu were determined in the rainwater samples collected at Hallen and Midger Woods (Table 38). At both sites the greater proportion of the metals was found to be associated with the residue i.e. > 0.45 µm particle size. In the Hallen sample, both the residue and < 0.45 µm fractions were found to be the more contaminated - having higher levels of all four metals than the Midger sample, as expected in view of Hallen's proximity to the Avonmouth Pb-Zn smelter. Of the Hallen "dissolved" or < 0.45 µm fraction most of the Zn and all of the Cd were associated with organic matter, colloidal and mineral materials, as was some of the Pb and Cu. For the Midger "dissolved" fraction, all of the Cd, and some of the Zn and Pb were associated with the organic matter and colloidal and minerals, but for Cu very little was released by the acid attack compared to the pH 3 treatment.

Despite being a rural site, at some distance (approximately 28 km) from the Avonmouth smelter, the Midger particulate fraction is highly contaminated (Zn 1406.0, Cd 41.6, Pb 1580.7 and Cu 133.0 µg/g). The contamination may be due to the proximity of the A46 (approximately 0.5 km northeast of the centre of the wood), or from the City of Bristol (22 km south west of Midger). Since the Zn level was high, as well as that of the Pb, the Midger site may be contaminated, as a result of a combination of severe pollution from the smelter and strong north-easterly winds at the time of collection. If the metal levels at Midger are influenced by the smelter, the plants at Midger may show an enhanced

tolerance to subsequent exposure to any of the metals (140), when compared to more isolated sites.

The results indicate the importance of "wash-out" from the atmosphere by rainwater - especially in the removal of particulate matter.

Luckett Mine drainage

Zn and Cu were determined in the drainage water collected at Luckett (Table 39). Contamination of the water sample with the metals is not surprising, in view of the copper mining activities which were carried out in Luckett until 1952. The metals existed mainly in a form which passed through the 0.45 µm filter. The increased Cu level after passage through the filter could be due to contamination of the filtered sample, possibly from metal leaching from the filter.

c) Sediment sample analysis

Caradon and Mineries Pool samples

The results in Table 40 indicate that the Mineries Pool sediment was highly contaminated with Pb, reflecting the history of Pb mining in the Mendip Hills. Due to the high metal content of the sediment the samples were submitted to a series of dilution steps, before measurement of the analytical signals. The absence of a decreasing metal concentration trend with decreasing size fraction may have resulted from errors in the dilution procedure. In general, the results show that the Pb was mainly associated with particles whose dimensions were between 0.1 and 5 µm - approximately one third of the metal in this fraction was particulate-Pb (i.e. > 0.45 µm), possibly consisting of Pb-organic complexes or particles of mineral matter (the organic material being derived from the flora and fauna inhabiting the pool and the mineral matter, such as galena, from the ore-washing procedures used in the seventeenth and

eighteenth centuries). As expected, the Pb level increased with decreased pH. Metal passing through the 0.45 µm filter was found to be principally ASV-labile at pH 3 (Figure 33), i.e. the metal was mainly associated with metal-organic complexes. In the < 5 µm and < 0.1 µm fractions a greater proportion of the Pb was desorbed from colloids. Very little of the Pb present in the samples was labile at pH 7.

The results demonstrate the importance of pH change in the aquatic environment - a decrease in pH causing the release of metals into the water from the sediments may result in toxic conditions for planktonic organisms and in pollutants being carried downstream.

The results for the Caradon sediment, fractionated by pH and filtration, are given in Table 41. The results show that the Caradon sediment was contaminated with Cu, as expected due to the Cu-mining activities previously carried out at Caradon. Because of the high Cu level of the sediment, dilution of the samples was again necessary, but not to the extent required for the Mineries Pool samples. Table 41 indicated that the Cu concentration decreased with decreasing size fraction and, in general, increased with decreasing pH. Approximately half the Cu present in the sediment was associated with the particulate fraction (i.e. > 0.45 µm), the remaining Cu existed mainly in a form which passed through the 0.1 µm filter. The Cu in each of the size fractions was found to be present as both labile and bound metal - approximately half of the Cu in the samples was ASV-labile at pH 3 i.e. present as metal-organic complexes while much of the remaining Cu was desorbed from colloids at pH 5. In comparison with the metal released by the pH 3 buffer, only a small quantity (from 2 to 3%) of the Cu was further released by acid digestion. Little of the Cu, then, was present as metal bound so firmly to dissolved organic, colloidal or mineral material that an acid attack was required for metal release.

The spurious acid digest and pH 3 values of the < 0.1 µm fraction may be due to dilution error, or sample contamination.

The metal levels of the acidified Caradon water sample (Table 36) are much lower than those of the sediment. The higher sediment values probably result from secondary deposition i.e. the enrichment of the sediment by the deposition of species such as $CuCO_3$, $Cu(OH)_2$.

Garston Dock sediment sample

Tables 42-45 show the distribution of Zn, Cd, Pb and Cu in > 40, 20-40 and < 20 µm size fractions. The sediment consisted of a range of particle sizes. The smallest particle sizes i.e. < 20 µm formed only a very small proportion of the total. Therefore, any metals associated with this fraction, however large the metal value, will not greatly affect the total concentration in the sediment. The concentrations of Zn, Cd, Pb and Cu showed a general trend of increasing with decreasing particle size for samples 1 and 2 and also sample 4 for Zn and Cd, i.e. the metal concentration was highest in the < 20 µm fraction. The reverse was found for Zn and Cd in sample 3 - increasing metal concentration with increasing particle size. For Pb and Cu in samples 3 and 4, the highest metal concentration was found in the 20-40 µm size fraction.

Any relationship derived between particle size and heavy metal content may be due to the fractions containing different minerals. Since Zn and Cd, and Pb and Cu, showed similar trends in their associations with the different size fractions, the presence of different minerals in the different samples may be the cause. The organic content of the sample would also have an influence, for example, it has been sugested that Pb and Cu form more stable complexes with humic acid than Zn and Cd (296,297), which may explain the similar trends exhibited by

Pb and Cu and by Zn and Cd.

Although in samples 1 and 2 the highest metal concentrations determined were in the < 20 µm size fraction, this fraction was a minor portion of the sediment. The > 40 µm fraction was the major portion of all four samples, and was also generally associated with high metal concentrations. Thus for these two reasons the > 40 µm fraction is very important.

The heavy metal contamination found in these samples is not surprising in view of the industrialised nature of Merseyside and in view of the fact that waste dumping had been previously permitted at Garston Docks.

Ystwyth rock sample

Table 46 shows that the rock sample was contaminated, as expected from the area's past association with Pb-Zn mining. Some of the metal present was in a labile form and was easily leached from the sample by a mild extractant (0.2 M ammonium acetate, pH 7). The Zn and Pb concentrations were much higher than those found in the sediment samples (Tables 31, 33 and 34) extracted with nitric acid. Table 46 thus demonstrates the problems encountered with the dumps of mine waste - metal can easily be leached from such areas by rainwater running over or seeping through the dump and so eventually reaching the river water (even though mining activities ceased over 90 years ago).

The effect produced by more acid solutions is also demonstrated in Table 46. The acid digest of the pH 7 leachate caused an 11% increase in the labile-Pb concentration and almost doubled the labile-Zn concentration. The results indicate, in some small measure, the problems that can result from acid mine drainage.

Slag samples

Table 46 shows that the slag samples were contaminated with Cu and Zn and to a lesser extent Pb.

The Roman smelting processes were wasteful, leaving a high proportion of Pb in the slag (298). Modern techniques leave slag with metal contents of the order of 0.05 to 0.1% w/w (127). The relatively low metal contents of the samples indicates that the slag must have been of Roman origin and then resmelted and reworked on later occasions by other miners.

d) Soil sample analysis:

Contaminated peat samples/ferric hydroxide samples

The results obtained from the sequential extraction (reducible before organic fraction) of Pb/Cu peat are presented in Table 48. From the table it is apparent that the Pb and Cu were not extracted by only one extractant and a proportion of the metal (22% Pb and 8% Cu) remained in the residue even after the use of four successive extractants.

Soil Zn may be found in any of the five fractions (116). In the present study the Zn extracted from the peat was associated entirely with the organic phase. No Zn was added and the level present was found to be slightly above the median concentration of 90 µg Zn/g of soil (but well within the range of Zn soil concentrations of 1 to 900 µg/g) (8). Under these conditions the organic "sink" would not have been saturated and considering the organic nature of the sample, the Zn-organic association is not unexpected. The addition of metals at high concentrations obviously did not cause a substitution of Pb or Cu for Zn in the organic fraction. Thus, the Zn present in this phase was firmly bound, especially since Slavek, Wold and Pickering suggested that Cd and Zn humate complexes are less stable than those of Cu and

Pb (237).

The recovery of metals during a sequential extraction procedure can be judged by comparing the summed total of each extraction with the independent total metal analysis performed on a separate sub-sample (235). The total Zn recovered by sequential extraction agreed well with the value obtained by acid digestion (HF/HNO_3).

In contrast to the Zn, the Cd found in the sample was present in the exchangeable (50%) and carbonate (50%) fractions. The results agree with those of Harrison, Laxen and Wilson, who observed Cd in the initial extractions of both street dusts and soils (235).

The total Cd recovered by sequential extraction agrees well with the acid digest result. No Cd was added to the peat, the Cd may therefore be present as either a contaminant of the peat or more likely of the analytical process. This may explain its different distribution in comparison with Zn, since both Slavek et alia (237) and Esser and El Bassam (82) regarded Cd as being more tightly bound by organic substances than Zn, however due to the polyfunctional nature of humic acids, extended metal-humate chains can be formed. Experimental conditions can influence the amount of each type formed, hence views differ on the relative order of stability for metal humate complexes (237).

Contamination or sample loss can be a problem in sequential extraction schemes, as a result of the number of steps involved. Loss of sample was found to be a source of error in this study, resulting from the "bubbling" effect produced when H_2O_2 was added to samples of high organic content. The problem was overcome by adding the H_2O_2 in 1 ml aliquots. Difficulties were also experienced in obtaining reproducible results due to the natural degree of variation in metal concentrations resulting from inhomogeneities in the sample.

Both Pb and Cu were recovered in every fraction and both were

predominantly associated with the reducible phase (41% Pb and 53% Cu). For Pb, the reducible fraction was followed by the residual (22%), carbonate (19%), exchangeable (11%) and organic (7%) fractions. For Cu, the sequence was carbonate (25%), exchangeable (14%), residual (8%) and organic (1%), As noted earlier both Pb and Cu are considered to form strong metal-humate complexes. Proposed sequences of the relative order of stability for metal humate complexes, include (237) :-

i) Cu >> Pb > Zn

ii) Pb > Cu > Ni > Cd

iii) Cu >> Zn > Pb.

Indeed, Slavek et alia observed that the electrolyte extractants (NH_4NO_3, NaCl, $MgCl_2$, $CaCl_2$) displaced only about half the absorbed ions which they suggested was indicative of a high proportion of Cu and Pb ions becoming coordinated to the organic phase (237). Harrison, Laxen and Wilson also suggested that the Pb-organic interactions may be of importance (235). Similarly Hidebrand and Blum (299) and Zimdahl and Skogerboe (300) considered the Pb-organic association to be significant. Other workers have found Pb to be absorbed by humic acid and concentrated in humus rich material (51). Harrison et alia also reported the Cu predominantly in the organic phase. The preference of Cu for the organic phase as well as the importance of the residual phase is often observed in sediments and soils (235). Thus, in view of the organic nature of the peat sample fractionated in the present study, the minor role played by the organic fraction was unexpected. Loss of sample could not explain the sequences determined, since the acid digest and sequential "totals" were similar. It is possible that the reducible extraction procedure is too severe for the type of sample studied - this would explain the predominant reducible-associations found. Maher (233) observed that the Fe/Mn and organic fractions were not well defined, especially for

organic rich sediments and this problem may arise in the present situation.

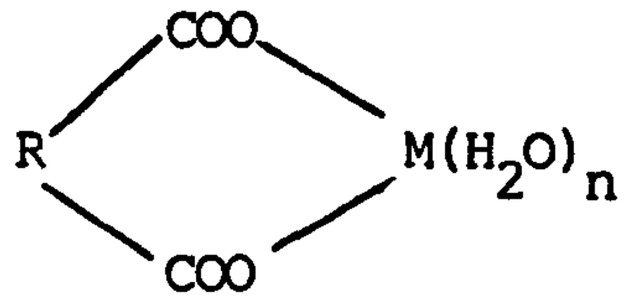
The fractionation scheme was repeated, using a Cu-peat sample and a modified organic extraction before the reducible fraction (Table 49). The organic extraction procedure was modified (0.002 M HNO_3 instead of 0.02 M HNO_3) to minimise interference with the reducible stage.

From Table 49, the sequence of metal associations was found to be :-

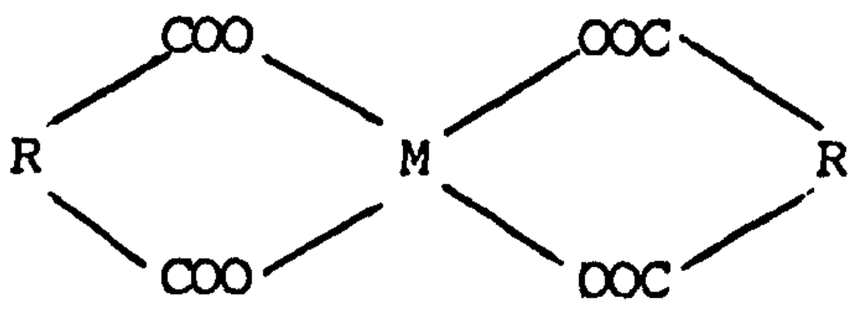
I) organic (44%), carbonate (27%), reducible (16%), exchangeable (10%) and residual (3%),

II) organic (39%), carbonate (29%), reducible (21%), exchangeable (7%), residual (4%) and soluble (1%).

The metal was now mainly associated with the organic fraction possibly as



or



complexes (301,302), although the reducible fraction was still found to play an important role.

Table 49 also shows the results obtained when a water-soluble fraction is added to the sequential extraction procedure.

The shortcoming of using water as an extractant is the differing solubilities of univalent and diavlent ions. Processes that are responsible for changes in the relative and total amounts of dissolved constituents that occur with increasing water content are exchange reactions in which dissolved divalent cations replace adsorbed univalent

cations. Thus, in assessing the absolute water-soluble metals, underestimation will result. Nevertheless, the soluble metal contents represent the most readily available portion for plant uptake and its significance should not be disregarded (239).

The soluble fraction (Table 49) was found to be of secondary importance - containing only 1% of the extracted Cu. It is well established that DDW is a poor extractant for most metals (177). After application of the soluble fraction, some change in the Cu distribution of the exchangeable and carbonate fractions occurred, but their relative importance in the metal-fraction sequence remained the same. The soluble and exchangeable contents generally represent a minor fraction of the total metal concentration of the solid (241,239), but are significant in that these fractions indicate the available portion of metals which will affect vegetation (239).

The comparison of HF/HNO_3 and HNO_3 acid digests (Table 49) indicates that the former is the more effective extractant, as expected due to hydrofluoric acid having the property of decomposing silica. If HF is used in conjunction with nitric, hydrochloric or perchloric acid, the total decomposition of silicates is possible. Anomalies in the total metal by sequential extraction and by acid digestion can result from metal loss during the "washing" stages of the sequential extraction, as well as the problems outlined earlier.

Although samples I and II were found to have the same relative order of importance of the metal-fraction associations, differences in their metal contents were observed. This may result from the problems discussed previously (i.e. contamination, loss or inhomogeneity of sample) or due to errors in dilution - necessary for analysis by DPASV in particular. Figure 36 is a voltammogram of 0.1% v/v Cu peat (exchangeable fraction).

Table 50 presents the results of the "4 metal" peat fractionation. The sequences determined are :-

Zn

organic (93%), residual (4%) and reducible (3%)

Cd

organic (33%), soluble (19%), exchangeable (17%), carbonate (14%), reducible (12%) and residual (5%)

Pb

organic (38%), exchangeable (23%), reducible (20%), residual (16%), carbonate (3%) and soluble (0.1%)

Cu

organic (71%), reducible (21%) and residual (8%).

The four metals were added to the peat as 0.1 M solutions. Thus, the concentration of metal added increased in the order Cu ~ Zn < Cd < Pb. A high level of Pb was determined in the peat sample as expected, but much less Cd and Zn were taken up by the peat compared with Cu - Cu almost invariably forms stronger complexes with most organic ligands than other transition metal ions (5,70) and the result reflects the greater affinity of humic acids for Cu (237).

Not surprisingly, the organic phase was the dominant phase for each of the four metals, the absolute concentrations in this fraction followed the order Pb > Cu > Zn > Cd. At such high metal concentrations, saturation of the organic "sink" is possible and the presence of metals in the carbonate and reducible phases may be indicative of this situation.

In the sequential extraction scheme used it is easy to envisage mobility and bioavailability decreasing approximately in the order of the extraction procedure from readily available to unavailable. For instance, the pH of the first extractions at pH 7 is representative of natural waters. Metals occurring in the carbonate and reducible phases can be

considered moderately available, while the metals occurring largely in the organic and residual phases is of very limited availability. The value of complex formation in soil solution is not that the chelates are absorbed but that the total concentration in solution is increased and thus increases the possible flux of metal towards a root surface, where free ion concentration is maintained by complex dissociation (145).

The Cu and Zn showed a similar fraction distribution, with no metal being present in the more available fractions i.e. soluble, exchangeable and carbonate. For Cu the reducible fraction was of greater importance than for Zn (possibly resulting from saturation of the organic sink). The Pb and Cd were found in all fractions, but 50% of the Cd was located in the more available phases (soluble, exchangeable and carbonate), while for Pb this value was only 26.1% and the reducible and residual fractions were of greater significance. Thus, the relative order of availability is Cd > Pb > Zn > Cu. Harrison, Laxen and Wilson found a similar order - Cd > Pb ~ Zn > Cu - in their study of street dusts and soils (235). The Cu is strongly held on both inorganic and organic sites and in complexes with organic matter i.e. it is not readily available for uptake by plants (81). For Pb, strong absorption on the surface of clay minerals, organic colloids and the formation of insoluble Pb chelates with organic matter, render Pb relatively immobile (81). Zinc complexes more strongly with soluble and insoluble ligands than does Mn, Zn is generally not bound as strongly as Cu, only a very small fraction of which is present in soil solution as the free ion (145). Lum and Edgar found that the Cd contents of fractions could be deemed readily or partly available but do not normally exceed 50% of the total (236). The Cd and Zn bonding in humate complexes is suggested by Slavek et alia to be predominantly electrostatic (i.e. $RCOO^{-}M_{n}^{2+}$) (237) and therefore displaceable by competing cations.

The residue,remaining after the four preceding extractions,consists essentially of detrital silicate minerals, resistant sulphides and a small quantity of refractory organic material (241). The results highlight and indicate the importance of the residual fraction - the association with the residual phase reduces the significance of the non-residual phases in less contaminated samples and illustrates the difficulty in distinguishing between background and anomalous levels of trace metal contamination when only a total metal analysis is performed.

Table 51 shows the results obtained from the fractionation of Pb contaminated ferric hydroxide determined by DPASV and FAAS. The Pb was located in all the fractions :-

	Percentage of total Pb			
Fraction	DPASV I	AAS I	DPSAV II	AAS II
Soluble	3	2	3	2
Exchangeable	0.03	0.04	0.03	0.03
Carbonate	44	38	38	39
Organic	26	31	5	6
Reducible	8	13	53	52
Residual	19	16	1	1

In view of the nature of the sample, little carbonate or organic material would be expected - therefore the Pb contents of these fractions must result from either attack by the carbonate and organic fraction extractants on the reducible phase, or from the saturation of the reducible (i.e. $Fe(OH)_3$) sink. When the organic extractant is applied prior to the reducible, the organic phase together with the carbonate phase were the dominant fractions. However, when the reducible extraction was carried out first, the reducible phase dominated. Thus, the extractants

used for the organic phase do attack the $Fe(OH)_3$-Pb association.

The Fe-Mn oxides have a strong scavenging efficiency for trace metals (234) which is illustrated in the present study by the high Pb concentrations found in the ferric hydroxide phase.

The DPASV and FAAS analyses gave similar results for samples I and II and their sequences of relative importance were similar. Differences in the values obtained are most likely to have resulted from errors in dilution - a procedure it was necessary to repeat several times despite a reduction in sensitivity (from 10 μA to 0.1 mA current, and from 120s to 60s deposition time). Figure 37 is a voltammogram of the Pb contaminated ferric hydroxide (exchangeable fraction).

SRM 718 Orchard leaves

Table 52 depicts the results of the fractionation of SRM 718 orchard leaves determined by FAAS, and compared with the NBS certified values.

The Zn was found in all the fractions, except the reducible, as follows :- Organic (40%), exchangeable (25%), residual (17%), carbonate = soluble (9%).

The Cd was found only in the exchangeable fraction.

The Pb was located in most fractions, except soluble and exchangeable, as follows :- Reducible (45%), residual (22%), carbonate (19%), and organic (15%).

The Cu was found only in the organic (70%) and carbonate (30%) fractions.

Although the Tessier et alia scheme (241) was proposed for the fractionation of sediments and later for street dusts and soils (235), the fractionation of a standard reference material, such as SRM 718, gives an indication of the accuracy of the procedure (303). Furthermore, the scheme provides a means of determining the mobility of the metals

within the plant sample - i.e. whether they are strongly bound (e.g. residual, organic) or easily leachable (soluble, exchangeable), an idea of the nature of the binding ligand can also be obtained :-

fraction (Tessier)	plant fractions	Reference
Soluble	free ions	304
↓		
Exchangeable		
↓		
Carbonate	some proteins	305
	pigments	306
	organic acids	307
	monosaccharides	308
↓		
Organic	pectin	309
	hemicellulose	310
↓	∝-cellulose	310
	proteins	311
	amino acids	311
Reducible	∝-cellulose	310
	∝-cellulose	310
↓	lignin	312
Residual	Ash	

The plant fractions do not correspond exactly to the Tessier et alia fractions - most plant substances are hydrolysed in the presence of a hot mineral acid.

From Table 52, the mobility of the metals decreases in the order Cd > Zn > Cu > Pb. The organic fractions were the most important for Zn and Cu (as would be expected from their enzymic roles); some Cu was also found in the carbonate fraction (i.e. pigments etc.) possibly due to the presence of Cu in plastocyanin. The "Zn-carbonate" association may result from the interaction of Zn with organic acids (49). The

non-essential Pb was mainly located in the cell-wall fractions. The Cd level determined was higher than the FAAS and NBS values and thus the presence of Cd in the exchangeable fraction must be due to contamination of this phase.

The metal levels determined in general agree with the NBS value - discrepancies resulting from the problems outlined earlier. The voltammogram obtained by HMDE-DPASV showed an interference, probably resulting from the presence of organic material. A stronger acid attack (such as perchloric acid) is required for satisfactory voltammetric analysis.

Hallen and Wetmoor Woodland litter samples

Table 53 (a & b) gives the results obtained from the fractionation of woodland litter samples determined by DPASV (and Zn by FAAS).

As expected the litter sample collected from Hallen contained higher levels of all four metals, than the Wetmoor sample - i.e. the metal levels were greater by a factor of 19 for Zn, 5 for Cd, 62 for Pb and a factor of 8 for Cu than the Wetmoor sample, due to the proximity of Hallen to the Avonmouth Pb-Zn smelter.

The Zn present in the two samples was found to be associated with the fractions in the following order :-

Hallen - residual (64%), reducible (22%), exchangeable (8%), organic (4%) and carbonate (2%).

Wetmoor - reducible (48%), residual (36%), exchangeable (9%), organic (5%), carbonate (1%) and soluble (0.6%).

The sequences are similar, with the residual phase being the more significant for the more polluted site - reflecting the tendency for the contaminated metal to become associated with the residual phase, thereby reducing the significance of the non-residual phases. A slightly

greater proportion of the Wetmoor Zn was found in the more 'available' fractions i.e. 10.6% compared to 10% for Hallen. Organisms found inhabiting the Hallen site are probably Zn tolerant in order to withstand a readily available Zn concentration of 391 µg/g (cf. Wetmoor the value is 19 µg/g).

For Cd the sequences are :-

Hallen - organic (48%), exchangeable (25%), residual (23%), and carbonate (3%)

Wetmoor - organic (60%), residual (33%) and exchangeable (8%).

The organic phase is the most important at both sites - the metal in this phase is not readily available for plant uptake. The residual phase also contains much of the Cd at both sites - which again is not readily available. But 28% of the Hallen Cd is readily available compared to only 8% of the Wetmoor metal. Organisms at the Hallen site must be able to tolerate 41 µg/g of Cd, whereas those at Wetmoor need to be able to tolerate 3 µg/g.

The Pb sequences for the two sites are :-

Hallen - reducible (51%), organic (21%), residual (20%), exchangeable (6%) and carbonate (2%)

Wetmoor - residual (29%), exchangeable (27%), organic (18%), reducible (15%), carbonate (8%), and soluble (3%).

The sequences differ for the two samples, with Pb in the more contaminated sample being found mainly in the "not readily" available fractions. But 8% of the Pb in this sample is readily available - amounting to some 586 µg/g. For the Wetmoor sample there is a greater proportion of Pb in the exchangeable fraction - but the readily available Pb is 47 µg/g.

Finally, the Cu sequences are :-

Hallen - organic (70%), reducible (27%), residual (7%) and carbonate (2%)
Wetmoor - residual (64%), organic (21%) and reducible (15%).

The Cu at both sites is relatively unavailable, being bound mostly by organic matter or Fe and Mn hydroxides.

The mobility/availability of the metals decreases in the order :

Cd > Zn ~ Pb > Cu Hallen

Pb > Zn > Cd > Cu Wetmoor

The Hallen sequence is similar to that determined for the contaminated peat (Table 50), i.e. Cd > Pb > Zn > Cu, and Harrison et alia (235) found the same sequence (i.e. Cd > Pb ~ Zn > Cu) for metal contaminated street dusts and soils. As expected, Cu is the least available of the metals due to the ability of Cu to form strong associations with clay, organic matter and hydrous oxides (70). Contamination from the Avonmouth smelter has increased the bioavailability of all four metals (but Cd, Pb and Zn in particular) at the Hallen site. Because of its organic nature, the litter has retained much of the contaminating metal found at the Hallen site - the absolute concentrations of this fraction follow the order Pb > Cu > Zn > Cd. The scavenging effect of Fe and Mn hydroxides is also demonstrated by the results.

The Wetmoor sample differs from the Hallen sample in the relative bioavailability of the non-essential Pb and Cd (both Cu and Zn have the same relative availability as in the Hallen sample). The absolute concentration of metals in the organic fraction is also different i.e. for Wetmoor the order is Cd > Cu > Pb > Zn. The differences between the two samples may be caused by the degree of contamination received or due to differences in the nature of the litter i.e. there is an increase in the available exchange sites per unit dry matter for more highly decomposed litter (313). Since heavy metals are strong toxicants to decomposer communities (132), the litter at Hallen is likely to be less

decomposed that that found at Wetmoor, despite both woods being deciduous.

Inman and Parker reported that heavy metals increased in litter with time (133), and the results shown in Table 53 (a & b) demonstrate the role of litter as a sink for heavy metals. High levels of organic matter in the litter and top few cm of the soils bind the contaminating metals in non-leachable forms and by buffering at near neutral levels allow precipitation of the hydroxy-carbonate solid phases (234).

In general, the sequential extraction totals agree well with the acid digest values. In both samples, the summed Zn totals show some Zn loss compared with the acid digest values - possibly as a result of adsorption on the container walls prior to analysis by FAAS because of high pH values.

Luckett and Callington soil samples

Table 54 (a & b) clearly indicates that both the Callington and Luckett samples were contaminated with Cu, Pb and Zn and to a lesser extent by Cd - reflecting their histories as areas of metal mining activity.

Table 110 gives the percentages of total metal found for each fraction.

The summed total by sequential extraction is less than the acid digest values - mainly due to sample loss on the addition of H_2O_2 (a problem mentioned earlier).

Table 110 : Percentage of total metal found, in Luckett and Callington soil samples

	% of total Zn					
Fraction	C_1	C_2	C_3	L_1	L_2	L_3
Soluble	1	0.2	-	2	-	-
Exch.	16	20	15	2	0.3	-
Carbonate	1	2	1	-	-	-
Organic	21	17	11	-	0.7	-
Reducible	15	14	7	-	0.7	1
Residual	46	46	66	96	98	99

	% of total Cd					
Sample	Sol.	Exch.	Carb:	Org.	Red.	Res.
C_2	-	50	50	-	-	-

	% of total Pb					
Fraction	C_1	C_2	C_3	L_1	L_2	L_3
Soluble	-	-	-	-	-	-
Exch.	-	1	3	-	-	-
Carbonate	-	-	-	-	-	-
Organic	7	21	44	-	-	-
Reducible	24	3	30	-	-	-
Residual	68	75	23	100	100	100

Table 110 continued.

	% of total Cu					
Fraction	C_1	C_2	C_3	L_1	L_2	L_3
Soluble	0.5	0.1	-	1	-	-
Exch.	1	2	0.3	2	-	0.3
Carbonate	0.1	0.4	-	-	-	-
Organic	39	35	29	6	5	3
Reducible	15	7	10	0.7	12	13
Residual	45	55	61	90	83	84

C_1 = Callington soil (at a depth of ~8 cm)

C_2 = " " (~4 cm) beneath A. tenuis

C_3 = " " (~1 cm) beneath R. squarrosus

L_1 = Luckett soil (~0 cm)

L_2 = " " (~4 cm) beneath A. canina

L_3 = " " (~1 cm) beneath C. purpureus

The results obtained again emphasise the importance of the residual fraction (i.e. detrital silicate minerals, resistant sulphides and refractory organic material, resistant to dissolution) in reducing the significance of the non-residual phases. The metal in the residual fraction is of very limited availability. Thus, all the Pb determined in the Luckett samples is of limited bioavailability. The greater proportion of the Pb in the Callington samples is located in the residual fraction - a small proportion however is readily available.

Only a small proportion of the Zn found in the Luckett sample is readily available. The Callington samples show a more diverse Zn

distribution and between 16 and 22% of the Zn is present in the first three fractions. Plants grown on the Callington soil could, therefore be expected to contain a higher Zn concentration than plants collected from Luckett.

Much lower levels of Cd were found than of the other three metals. The Cd determined in the C_2 sample was readily available.

The majority of the Cu found in the Callington samples was located in the organic and residual phases. In samples C_1 and C_2, Cu was also associated with the first three fractions (i.e. readily available). For the Luckett samples Cu was mainly associated with the residual phase. The sample L_1 contained more Cu in the soluble and exchangeable phases than the other two Luckett samples.

In general the bioavailability of the metals follows the order :-

Zn > Cu > Pb for Callington

Cu > Zn > Pb for Luckett

Hallen soil sample

The results obtained in Table 55 show the fractionation of the soil collected from Hallen Wood. The soil was found to be contaminated with metal (Zn > Pb > Cd) - the levels of Zn, Pb and Cd were much greater than median soil concentrations of 90, 35 and 0.35 µg/g respectively. The Cu concentration was slightly higher than the median Cu concentration of 30 µg/g (8). Compared with the metal levels determined in the Hallen litter sample (Table 53a), the concentrations found in this soil are much lower - clearly emphasising the role of litter as a sink for heavy metals (234).

No Zn, Cd, Pb or Cu was found in the soluble fraction of the soil. The Zn was located mainly in the organic (37%) and reducible phases (37%), the remainder was found in the residual (22%), exchangeable (4%) and

carbonate (0.2%) fractions. Much of the Cd was located on the organic (40%) and exchangeable (56%) fractions. Unlike Zn, only 4% of the Cd was associated with the reducible phase. A greater proportion of the Cd is readily available compared with Zn. Taylor (118) also found that Cd remained soluble. Since any hazard to crops or the animals that eat them depends largely on the availability of an element for plant uptake, the ready availability of Cd is a cause for concern. A number of authors (6,22,55,117) have noted Cd accumulation by plants in contact with contaminated soil.

Both Pb and Cu were found in the last three fractions i.e. Pb reducible (47%), organic (42%) and residual (11%) and Cu, organic (56%), residual (32%), and reducible (11%). The bioavailability of the four metals, therefore, declines in the order Cd > Zn > Pb ~ Cu. Thus, compared with the litter sample, the sequence is similar but the soil Pb availability has declined - i.e. at the lower Pb concentration, the organic and reducible sinks retained the metal more effectively.

Vegetation growing on the Hallen soil would have to tolerate :-

i) readily available Zn concentrations of 63 µg/g and moderately available concentrations of 610 µg/g;

ii) readily available Cd concentrations of 14 µg/g; and

iii) moderately available Pb concentrations of 115 µg/g.

Somerset and Avon soil samples

Table 56 gives the total (acid digest), Zn, Cd, Pb and Cu concentration in soil samples collected around Bristol.

High levels of Zn, Cd and Pb were found in the soils collected from Shipham, Charterhouse and Bedminster. The Cu level was also high for the Bedminster sample, but was close to the median concentration of 30 µg Cu/g soil, for most of the other samples studied.

The Royal Fort Gardens' samples were relatively uncontaminated, but the samples collected from the Computer Centre site reflected the site's proximity to a car park - the Zn level was increased by a factor of 6, the Pb and Cu concentrations by a factor of 2 and Cd was present at a concentration of 1 µg/g.

The Shipham samples, as expected, were highly contaminated with Zn, Cd and Pb - the concentrations of which increased at a depth of 10 cm. At sites with high soil metal levels derived from underlying mineralised material, the metal level usually increases with increased soil depths. Under such conditions deeper rooted species may take up more or less metal than shallow-rooted species, depending upon the availability of the metal (135). However, the soil at Shipham has been disturbed by mining activity and the metal levels will vary accordingly. The metal levels found are so high as to suggest that much of the Zn, Cd and Pb will be available for plant uptake. From the fraction distributions found for Zn and Cd in the samples studied previously (Tables 53, 54 and 55), these two metals, in particular, are likely to be available. Vegetation growing on this site may well have developed mechanisms by which it can tolerate these metals. The metal concentrations determined in the Charterhouse samples were lower than those for Shipham (lower by a factor of 22 for Zn, 7 for Cd, 2 for Pb and 3 for Cu). In view of the high metal concentrations of these sites, plant uptake will occur - Cd in particular will be taken up, since the metal tends to remain in the exchangeable phase. Indeed Davies and Ginnever (20) found levels of up to 1.8 µg Cd/g (DW) in brussel sprouts, 0.6 µg Cd/g (DW) in potatoes and 2.5 µg Cd/g in root vegetables, grown on Shipham garden soils, which were factors of 8.2, 2.2 and 2.3 (respectively) greater than those for a control garden.

The Bedminster soil sample was contaminated with all four metals

(Tables 56 and 57), reflecting the area's past association with the manufacture of lead shot and brass. Again, plant uptake is likely. A further sub-sample of the Bedminster soil was studied by DPASV-HMDE and DPASV-RDE, Table 57 and Figures 38 and 39. Dilution of the sample was necessary. The HMDE results are similar to those obtained by FAAS - differences being due to sample inhomogeneity and dilution errors. It proved impossible to obtain values by RDE analysis - interference (probably from organic matter in the sample) being a major problem. Gunasingham and Fleet observed that the adsorption of electro-oxidation products is a major cause of variation in electrode performance (314). Florence noted that differential pulse peak currents are susceptible to interference by surface-active substances, and undetected adsorption/ desorption effects can lead to serious errors (315). The problem can be overcome by destroying the organic matter with perchloric-acid, for example.

"Quick" fractionation scheme

The "Quick" fractionation procedure was proposed to provide a rapid means of determining the degree of metal availability. From the results attained the scheme achieves this overall aim. But since it incorporates the same 'reducible' extractants, the scheme suffers from the same disadvantages as the Tessier scheme, in that, for samples of high organic content, metals are leached from both organic and reducible phases by these extractants. In order to overcome this problem, determination of the organic fraction before the reducible may again be necessary.

Table 58 shows the results obtained for "4 metal" peat. The four metals were found in all fractions. The Zn was associated mainly with the exchangeable/carbonate (36%) , reducible (33%) and residual fractions (30%). A small proportion was in the organic (2%) fraction. The Cd was

mainly in the first two fractions - exchangeable/carbonate (57%), reducible (42%), a very much smaller quantity being in the organic and residual phases (both 0.4%). The dominant Pb fraction was the reducible (44%) followed by exchangeable/carbonate (26%), residual (22%) and organic (8%). For Cu the sequence was exchangeable/carbonate (48%), reducible (35%), residual (11%) and organic (5%). Thus, despite the sample being of an organic nature, the organic fraction was found to contain the smallest proportion of Zn, Cd, Pb and Cu, i.e. the heavy metal retained by the organic phase was leached by the extractants used to fractionate the reducible phase. In future applications of the Quick scheme it is necessary to carry out the organic extraction prior to the reducible extraction. The absolute concentrations of metals in the organic phase decreased in the order Pb > Cu > Zn > Cd - the same order was obtained for 4 metal peat fractionated by the Tessier scheme (Table 50), and again reflects the greater affinity of humic acids for Pb and Cu compared with Zn and Cd (237).

The metal availability decreased in the order Pb > Cu > Zn > Cd for 4 metal peat fractionated by the Quick scheme. The sequence obtained by the Tessier scheme was Cd > Pb > Zn > Cu. The difference results from :-

1. The reducible fraction was extracted before the organic - which causes an apparent increase in the moderately available metal;

2. Citric acid is a more severe extractant than sodium acetate and therefore leaches some of the moderately available metal from the reducible phase.

Table 59 lists the results obtained for SRM Orchard leaves. The Zn, Pb and Cu are distributed among all four fractions. The Zn was located mainly in the reducible fraction (31%), followed by exchangeable/carbonate (27%), organic (22%) and residual (19%). The Pb was mainly

associated with the residual fraction (44%), followed by organic (22%), exchangeable/carbonate (20%), and reducible (14%). The Cu was also mainly associated with the residual fraction (37%). The remaining Cu was in the exchangeable/carbonate (25%), reducible (21%), and organic (17%). The mobility of the metals decreases in the order Zn > Cu > Pb - as found using the Tessier scheme. However, in comparison with the Tessier scheme the organic fraction was no longer the most important for Zn and Cu i.e. the metal content of the reducible fraction had increased at the expense of the organic fraction. The cell wall (i.e. residual) fraction remained the most important for Pb.

The Quick scheme provides a means of estimating the available metal quickly and since it avoids the use of H_2O_2, the problem of sample loss is reduced. The summed and acid digest totals were therefore in better agreement than was found for some of the Tessier applications and also applies to the NBS values and the Quick scheme totals.

e) Humic and Fulvic Acid interaction with heavy metals

As a result of the metal-organic associations observed in the previous section (i.e. Pb and Cu having a greater affinity for the organic fraction compared with Cd and Zn), it was decided to study the interactions of the metals with humic (HA) and fulvic (FA) acids.

Table 60 shows the elemental composition of (a) HA chemically extracted from Levington's Universal Mixture and (b) a commercial preparation. From the table, it can be seen that the nitrogen, carbon and hydrogen content of the chemically extracted HA is greater than that of the commercial preparation - the contents of nitrogen and carbon in particular are higher. Table 61 shows the elemental composition of the two FA fractions extracted from Levington's Universal Mixture. The nitrogen,

carbon and hydrogen values of F_1 are slightly higher than those of F_2. Fulvic acid has been found to contain more oxygen, but less carbon and nitrogen than humic acid (95). Tables 60 and 61 indicate a much lower carbon content for FA compared with HA, but the FA nitrogen content is slightly higher than that of HA. However, from Table 111, it is evident that, although there is some variation, the overall elemental distribution is similar in most humic substances (316).

Table 111 : Percentage elemental composition of some typical humic substances (316)

Type	Carbon	Hydrogen	Oxygen	Nitrogen	Sulphur
Soil HA	58	5	34	3	1
Lake Sediment HA	53	6	35	5	-
Coal HA	64	4	28	1.5	1
Soil FA	45	4	45	1	1
Water FA	46	6	45	3	-
Soil Humins	56	6	32	5	1

The C/H ratios for the HA and FA studied are given below :-

Humic Acid	C/H
"Levington's" 1	12.0
"Levington's" 2	11.6
Mean	11.8
"Aldrich" 1	11.1
"Aldrich" 2	11.5
Mean	11.3

Fulvic Acid	C/H
F 1 (I)	10.9
F 1 (II)	9.9
Mean	10.4
F 2 (I)	10.4
F 2 (II)	10.3
Mean	10.4

The C content of the HAs is a measure of the degree of condensation (286) and the Commercial HA, having the slightly lower C percentage, indicates that the Levington's HA may be in a greater humified/condensed state. Although the C percentage has increased, the H value is proportionally lower (i.e. C/H is 11.8 for Levington's, while for commercial HA C/H is 11.3), indicating more C-C bonds (humification end product would be graphite (286)). The C values for the FA studied being lower indicate that the FA is less condensed than the HA.

Humic substance molecules are complex (91,317), which, in view of their origin (i.e. biodegradation of organic substances (95,318)) is not surprising. Humic substances, and more specifically fulvic and humic acids, have been the subject of intense research (319), but remain controversial (91). A number of difficulties have been encountered in the study of humic substances. The nature of the solutions used for the extraction of humic material may have significant effects on the total nitrogen content and on the nitrogen distribution in acid hydrolyzates. Problems have also been encountered in the determination of oxygen-containing functional groups - the range of values reported for any specific group is considerable. There is always the possibility of auto-oxidation of humic substances in contact with air, during the isolation procedure. Furthermore, the fractions obtained from the

isolation procedure are not homogeneous - sequential extractions have been suggested, but the extractants often contain carbon, which becomes incorporated into the extracted material. Research is needed to improve methods for the analysis of oxygen containing functional groups in humic substances. Widespread discrepancies have been found in the determination of the molecular weight of humic substances, due to differences in origin, extractants, degree of purification. The non-homogeneity of the sample has been an obstacle in physical methods of characterisation - for example in thermal analytical methods the presence of proteins and carbohydrates are thought to be the cause of elevated aromaticity values. Similarly, carbon-dating has been beset with problems as a result of non-homogeneity (95). In the light of these problems, an investigation of the molecular weight and total functional group content was not carried out at this time. A future investigation is recommended, when time is available.

The UV analysis of the Levington's HA and FA shows a generally uncharacteristic spectrum. The intensity of absorption decreases as the wavelength increases. The higher optical densities at shorter wavelengths have been attributed to increased mobilities of π electrons over aromatic carbon "nuclei" and over unsaturated structures conjugated with these nuclei (316). The UV spectra of humic compounds of diverse origins are very similar in spite of differences in elementary composition, sedimentation characteristics and other properties (286) - the UV spectra obtained for the Levington's FA and HA were found to be similar to those obtained by Thorne (286) for sediment-HA and -FA.

The voltammograms (Figure 45 a & b) indicate the effect of standard additions of Zn, Cd, Pb and Cu on solutions of FA and HA. From Figure 45a, it can be seen that Zn was present in the FA solution, prior to the standard addition procedure. The Zn spikes showed little effect of FA

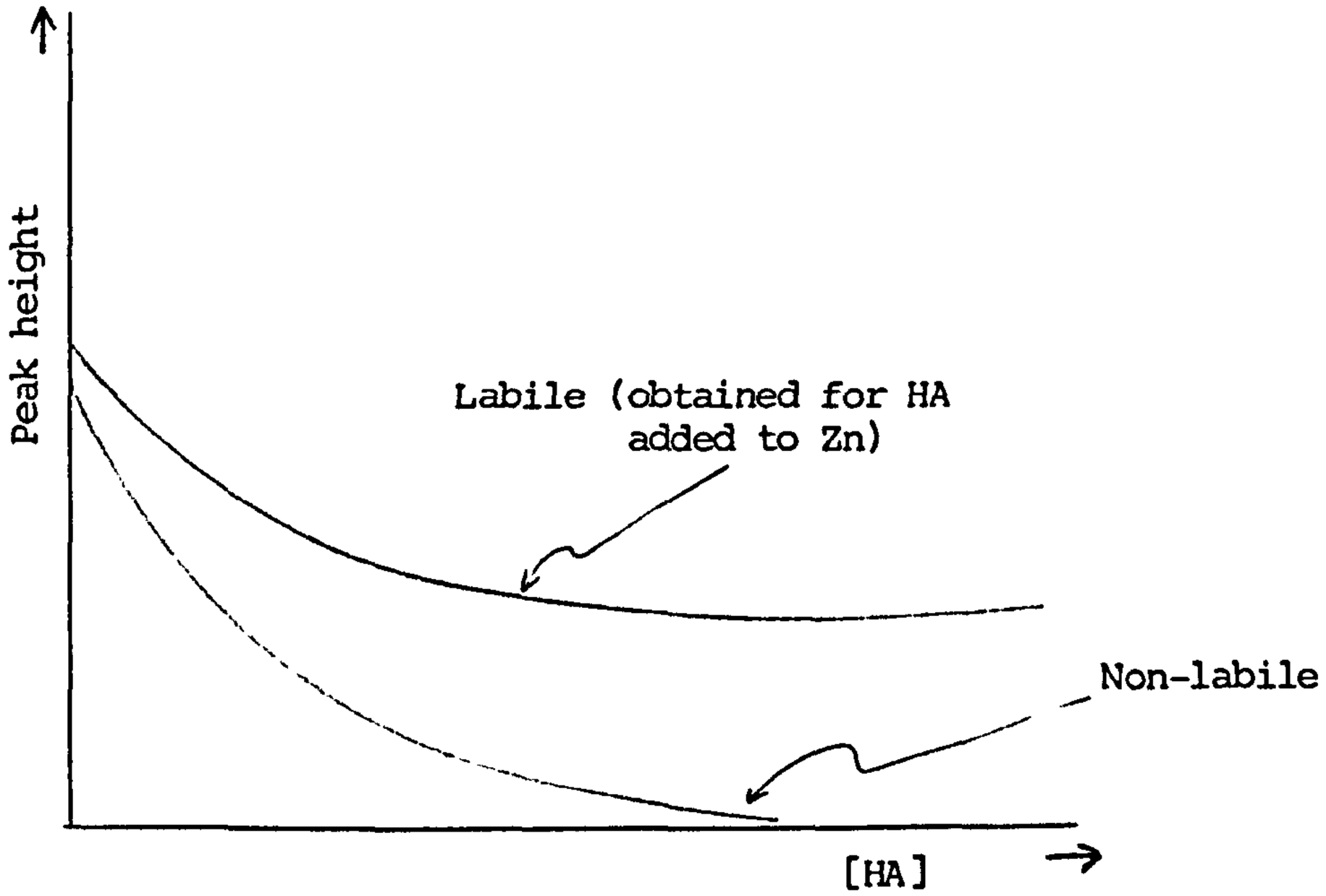
complexation. The Cd spikes were not affected by the FA, but both Pb and Cu showed reduced peak heights. From the voltammogram, the FA has a greater affinity for Pb and Cu than Zn or Cd.

The HA (Figure 45b) was at a greater concentration than the FA (i.e. 1.82 mg/ml for HA; 1.10 mg/ml for FA), and higher concentrations of the four metals were required to produce peaks. Despite this no Zn, and very small Pb and Cu peaks were visible. The Cd peak was the least affected by the presence of HA in the polarographic cell. From the voltammogram the HA has a greater affinity for Zn, Pb and Cu than for Cd.

In order to remove the possibility of intermetallic effects and to study the HA-metal interaction individually, the four metals were studied separately. The concentration of HA used was 0.07 mg/ml, while that for FA was 0.42 mg/ml.

The voltammogram in Figure 46 shows a decrease in the Zn peak height upon the addition of aliquots of HA, i.e. the Zn was bound to the HA. The change in peak height against volume of HA added is shown graphically in Figure 47. The peak height levels off after the concentration of HA has reached about 5.6 mg/l. This effect allows the lability of the Zn/HA complex to be determined. If the Zn/HA complex were non-labile, it is expected that the peak height approaches a zero value after addition of a large volume of HA i.e. after all the Zn had been irretrievably bound by HA. Thus, the complex formed is labile. A comparison of the theoretical titration curves for labile and non-labile complexes is shown in Figure 104.

Figure 104 : Comparison of labile and non-labile titration curves (theoretical)



The peak height for the labile complex is limited due to the larger size of the complex compared with that of the "free" Zn and hence diffuses more slowly to the electrode. The value of the diffusion coefficient for the complexed Zn (Dc) was found to be 1.44×10^{-7} cm^2s^{-1} (the value of Dnc i.e. the diffusion coefficient for the non-complexed Zn is 7.2×10^{-6} cm^2s^{-1} (288)). The accuracy of the value of Dc is limited, since the viscosity of a solution containing HA or FA is higher and adsorption effects at the HMDE will cause interference. Values for some diffusion coefficients are shown in Table 112a.

Table 112a: Some diffusion coefficients for metal-FA complexes

Complex	Dc (cm^2s^{-1})	pH	Reference
Pb/FA	2×10^{-6}	6	320
Cd/FA	1×10^{-6}	5.7	321
Cu/FA	7×10^{-7}	5.7	321

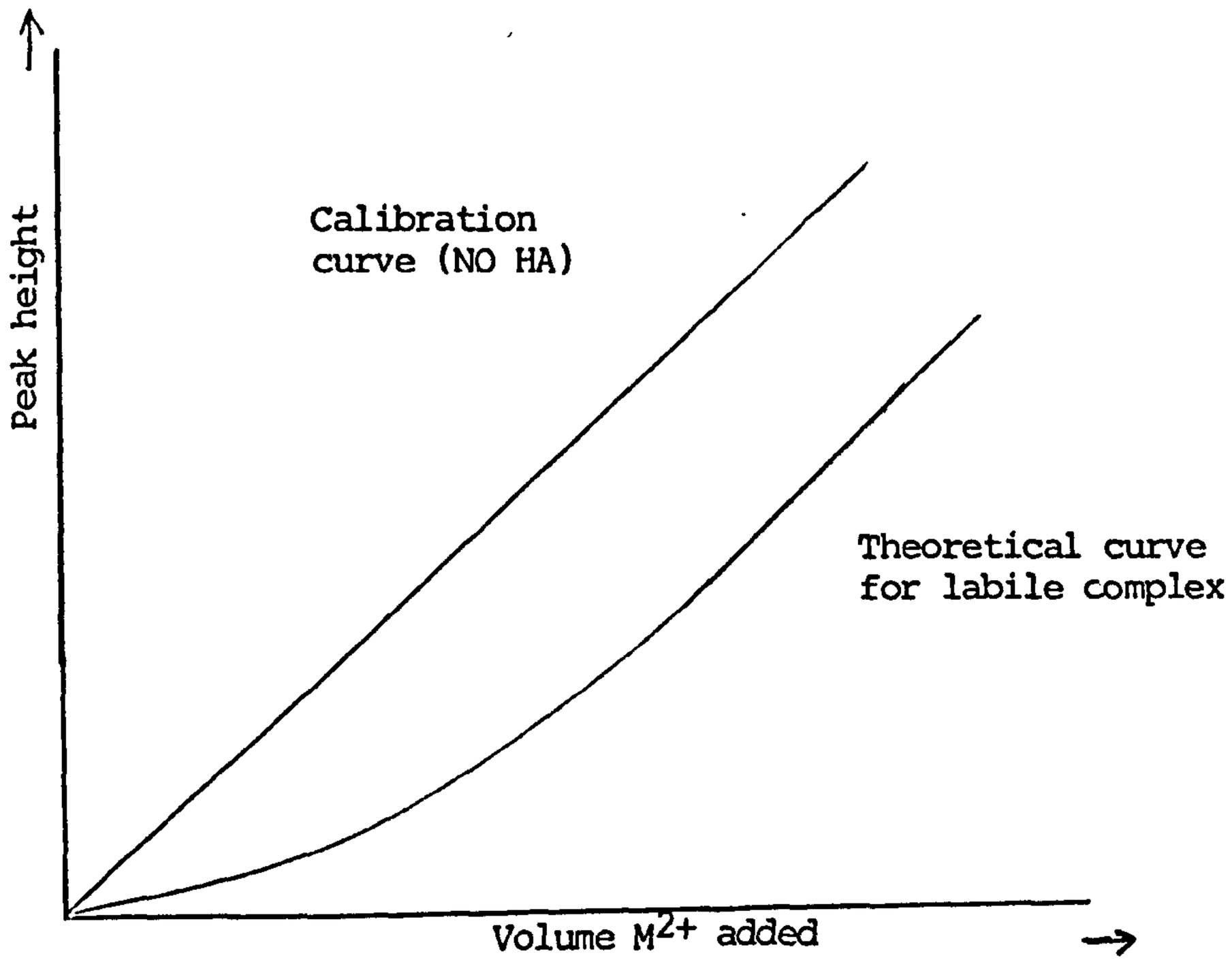
The values obtained in this work :

	Dc (cm^2s^{-1})
Cu/HA	1.40×10^{-6}
Cu/FA	1.89×10^{-6}
Pb/HA	2.60×10^{-6}
Pb/FA	2.50×10^{-6}
HA-Zn	1.44×10^{-7}

are of a similar order, but the Cu complexes were found to have greater Dc values. The low precision of Dc may in part be due to changes in the composition of the solution during the experiment, such as the formation of aggregates of humic material (320).

Figure 50 shows the effect of the addition of aliquots of Zn to a solution containing HA. The Zn peak height against concentration of added Zn is given in Figure 51. The curves for both the calibrating solution of Zn only and for the addition of Zn to HA have practically the same gradient. If complexation between the Zn and HA had occurred, it is to be expected that due to its larger size, the Zn-HA complex will diffuse more slowly than the uncomplexed metal, resulting in a decrease in current (i.e. peak height). An increase in gradient matching that of the uncomplexed metal would only occur after saturation of the HA chelating sites i.e. as in Figure 105.

Figure 105 : Theoretical curves for the calibration (i.e. no HA) and formation of a labile M^{2+}-HA complex



Since the titration and calibration curves (Figure 51) show almost the same behaviour, no complexation has occurred. The small differences between the two curves are attributable to the increased viscosity of the HA containing solution. Similar results were obtained on the addition of aliquots of Zn to FA (Figures 58 and 59), showing that no complexation occurred. Thus, no complexation resulted when Zn was added to HA or to FA, but did occur when HA was added to a Zn containing sample. Mancy and O'Shea (15), using ASV to study a Cd/FA system, noted similar results, i.e. a labile complex was formed when the metal was the titrant and a non-labile complex resulted when the metal was titrated with FA. Saar and Weber (322) suggested that when the FA molecules are in a concentrated solution, the molecules are crowded and therefore coiled. When the FA is added to the Cd solution, the subsequent mixing and dilution allow the

molecules to change shape and perhaps cause an increase in entropy, thereby providing the necessary energy for complexation. An already diluted solution of FA would not have this capacity. HA molecules may behave in the same manner.

Figure 52 shows the effect of Cd additions to HA and Figure 53 is a graphical representation of the Cd peak height against Cd concentration. The curves again show similar gradients - complexation did not take place. Figure 60 is a voltammogram of Cd additions to FA and Figure 61 shows the calibration and titration curves for Cd added to FA. The results are similar to those for Cd added to HA; complexation did not occur.

Figure 48 displays the effect of addition of Cu to a solution of HA. The peaks initially show broadening. Figures 48 and 49 indicate that a high concentration of Cu (500 ng/ml) is required before the Cu peak is off scale. In Figure 49, the titration curve initially follows a different gradient to that of the calibration (Cu only) curve, but gradually increases until the titration graph forms a slope beneath the calibration line. This region presents saturation of the HA bonding sites and the presence of excess Cu - the difference in gradient at this point is probably due to viscosity effects. If the Cu-HA complex were inert, then the observed current at the beginning of the plot would have been zero while towards the end there should be a break point, where the current increases linearly, yielding a slope equal to that obtained in the absence of HA (320). Such behaviour is not exhibited in Figure 49. The results, therefore, indicate the formation of a labile complex. Greter et alia (320) observed the same behaviour for Pb-HA/FA,Cu-HA/FA complexes using aquatic (freshwater) HA and FA. Indeed, in this study, labile Cu-FA (Figures 56 and 57), labile Pb-HA (Figures 54 and 55) and labile Pb-FA (Figures 62 and 63) were observed. However, at low metal levels the titration curve is almost linear, where the metal ligand

complex may be regarded as non-labile, before passing into an intermediate and then labile stage, as more metal is added, finally the gradient of the curve becomes steeper (Figure 49a) (321). The conditional stability constants for the complexes observed, were calculated :-

	KML (mol^{-1} l)
Cu-HA	9×10^5
Cu-FA	9×10^3
Pb-HA	1.88×10^5
Pb-FA	6.22×10^3

The stability constants are conditional, applying only to pH 7.0. The stability constants calculated for some metal/FA complexes, by other workers, are given in Table 112b.

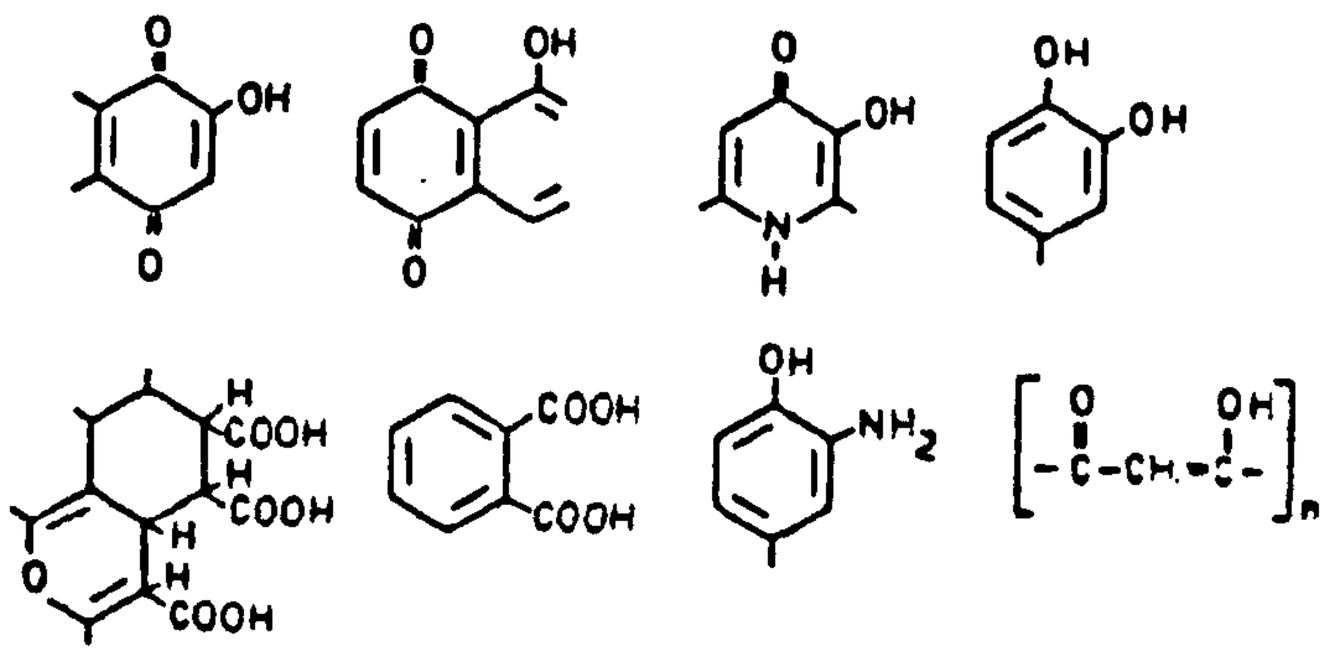
Table 112b: Some stability constants for metal-FA/HA complexes

Complex	Stability constant	pH	Method	Reference
Cd/FA	6.3×10^3	5	ISE	322
Cd/FA	2.3×10^3	5.7	DPP	321
Cu/FA	4.7×10^3	7	ASV	323
Pb/FA	1.2×10^5	6	DPP	324
Cd/HA	7.94×10^6	3 to 7	potentiometric titration	325
Cu/HA	7.94×10^8	"	"	325
Pb/HA	5.01×10^8	"	"	325
Pb/HA[a]	10.00×10^6	4 & 5	-	326
Pb/HA[b]	6.3×10^6	6.8	-	326

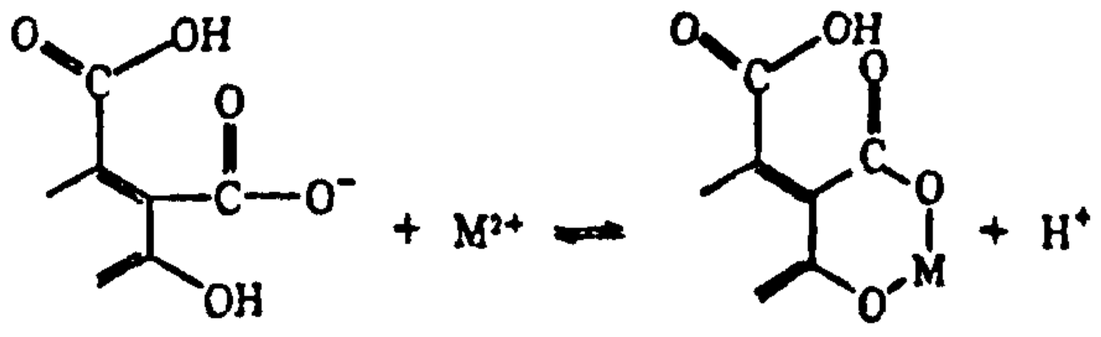
[a] soil derived HA
[b] Aldrich HA

Since metal complexation by humic substances results in the release of protons, metal complexation must inevitably be studied in competition with proton binding. It is therefore advantageous to use conditional stability constants to describe metal binding at constant pH (98). The stability constants calculated in this work are lower than those given in Table 112b;however metal-HA and metal-FA stability constants have been determined by a number of workers (Table 112band references 327 and 328), and the results vary widely. There is still considerable uncertainty about how to define the problem, which method to use and how useful such constants are once they have been determined. The main reason for this uncertainty is that the chemical structure of humic materials remains largely speculation (95). Structures commonly considered to be present in humic substances include the following (329) :-

Figure 106 : Structures considered to be present in humic substances (329)



It has been suggested that the binding of metals to humic acids involves chelation of a type similar to that observed with salicylic acid, i.e. :-



with the stability of the metal-humate complex being a function of the nature of the metal ion, the binding energy of the ligand functional groups, and environmental factors such as pH (93).

The numerous problems encountered in determining stability constants of metal-soil organic matter complexes also explains the wide variation found for stability constant values. For example, some of the approaches are those developed for the coordination chemistry of simple molecules and they apply in only a superficial way to complex macromolecules, such as HA and FA. Humic substances from whatever source are heterogeneous with respect to molecular weight, and a pH effect will determine the degree of ionisation of acidic groups and thereby the number of sites available for binding. Furthermore, several classes of binding sites may be present (e.g. Figure 106), in which case the site forming the most stable complex will be the first to react. The possibility also exists that HA and FA contain combining sites which are identical but react in such a way that binding at one site may accompany changes in pH or concentration of neutral salt.

The accuracy of the stability constant values depends on the assumptions of the molecular weight of the humic material. The values for the molecular weight of FA given in the literature lie mainly around 650 and for HA the range of molecular weight values is between 2000 to 10000, with a median value of 4000 (321).

Another potential source of error is adsorption of humic materials onto the mercury electrode (321,310,330,331). Very strong adsorption might distort the wave and cause a non-linear relationship between the stripping current and the bulk metal ion concentration (331) - ASV in particular is very susceptible to adsorption-induced errors, as the drop remains static for a long period of time (321). An increasing concentration of the humic material results in increasing coverage of the electrode and

hence decrease the rate of electron transfer. A second adsorption effect is caused by M^{2+} ions bound to adsorbed humic material. When the adsorption/desorption process is slow compared to diffusion and the rate of dissociation of any adsorbed M^{2+} complex is slow, some of the metal is unavailable for plating and hence the stripping current will be lower (331). Weak or moderately weak adsorption quite often does not cause abnormalities in polarographic measurements. The absence of such anomalies suggests that the linear relationship between the free metal ion concentration and the voltammetric current is not affected. Therefore, estimation of free metal ion concentration from the measured current is justifiable when a calibration curve is carried out under the same experimental conditions (210).

No abnormalities were observed for Zn, Cd or Pb, but at low Cu concentrations, the Cu peaks showed some broadening, when titrated with HA or FA (Figures 48 and 56). At higher Cu concentrations the broadening did not occur. The effect of adsorption of humic material is sometimes manifested by shifts of peak potentials. The determination of stability constants on the basis of peak potential shifts must take adsorption into account (210) - this method of calculation was not used in the present work.

From the results, the relative strengths of complexes of metals with both FA and HA follow the order

$$Cu > Pb > Zn \sim Cd.$$

A sequence that was found earlier, for the association of the four metals with the organic fraction. Other workers have reported that metals of the first transition series of the Periodic Table form complexes with humic substances, and that the order of stabilities of the different metal complexes follows that of the Irving-Williams series (93,95,237,325):

$$Pb^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+}$$

Stability constant data suggests that the hydroxamate linkages (-CO-N(OH)) may be partly responsible for the affinity of Cu^{2+} for some humic substances (332). Copper (II) ions are also able to promote deprotonation of phenolic groups and hence coordination (96). Since proton displacement is involved in the metal uptake prpcess, it can be concluded that the reactivity of cadmium will depend on the acid strength of the functional groups, hence behaviour could vary with the source of humic acid - and provides an explanation why HA and FA were not found to complex Cd and Zn in this study, but are known to form complexes with these metals (237). Organic matter possessing readily dissociated acid groupings should adsorb significant amounts of Cd over a wide pH range, whereas more basic groupings (e.g. -OH) may become more reactive only in alkaline media (93).

The results indicate the importance of HA and FA in 'regulating' metal availability for plant uptake. The above findings suggest that Pb and Cu are less likely to be available than Cd or Zn, in an organic rich soil. Indeed, Petruzelli and Guidi (333) found that wheat plants were only able to take up Cu from the weaker sorption sites of humic substances. The results are also relevant to the land application of sewage sludge - the organic matter of sludge, especially the humified fraction, may bind toxic metals and make them less available to injure plants in spite of raising the metal content of the soils above the natural background level (334). The preferential binding of Cu over Cd, illustrated by the above results, suggests that Cd may still be available for uptake by plants.

The work carried out here provides a means of verification for the metal-organic interactions determined earlier (Section 3 : Soils). A more detailed investigation of FA/HA-metal complexation will require the availability of model fulvic and humic acids (probably prepared synthetically) in order to prevent contamination by the other degradation products of plant organic matter. The model may be developed from the present knowledge of the organo-geochemist on kerogen (335), or by a controlled degradation of lignin (336). Preparation of synthetic model compounds would also offer a possible common source of humic or fulvic acids. At present comparison of the results obtained by different workers is difficult due to the various sources of the humic or fulvic acid (210,237,216,332) used. Humic-like models have been proposed, for example by Cariati et alia (96), who studied diaquabis-(2-6-dihydroxy-benzoate) Cu(II) and hexaqua M(II), bis(2,6-dihydroxybenzoate) dihydrate (where M = Mn, Fe, Co, Ni, Cu and Zn). It is hypothesised that humic materials consist of phenolic and benzene carboxylic acid "building blocks" (95). Synthetic model compounds could be developed using such building blocks in controlled condensation reactions.

During voltammetric experiments humic materials become adsorbed onto mercury electrodes, necessitating assumptions in the calculations of free metal ion concentrations from the observed currents (210). Furthermore, commercial instruments do not allow complete control of all parameters i.e. for the PAR Model 174 the differential pulse excitation wave-form consists only of a slow linear potential ramp upon which is superimposed fixed height voltage pulses. The pulse duration is 56.7 ms - the last 16.7 ms of which are used to measure the Faradaic current (337). Future investigations will require greater variability of these parameters. Ultrafiltration could provide an alternative method of study and has been used extensively to fractionate both organic material and trace

metal constituents (232) - Buffle et alia recommended sequential filtration for the separation of fulvic and humic acid from other material (338). The method appears to be the most promising for the investigation of such multiple-coordination interactions, but even here problems can be expected namely size selectivity, contamination and adsorption (232).

f) Plants : Metal fractionation studies

H. lanatus from Hallen and Midger Woods by Tessier Scheme

Table 62 shows the results obtained from the fractionation of H. lanatus plants hydroponically grown in Cd amended culture solution.

Cadmium was readily taken up into the roots of both Hallen and Midger plants - much less Cd was located in the shoots. The Hallen plants contained the greater concentration of Cd in the roots (46% more than the Midger population), but a lower concentration in the shoots (26% less). Tolerance to Cd by the Hallen plants is, therefore, at least partly related to the restricted translocation of the metal from root to shoot.

The Cd concentrations of the control plants are similar for the two populations, with the Hallen plants showing the slightly higher Cd levels. For both populations, the Cd content of the shoots is similar to the concentration found in the roots.

The Cd was found in all fractions for both roots and shoots i.e. from Table 113 the Cd was present as free ions, but also in association with proteins and/or organic acids, and the cell wall components. The Cd associated with the various fractions is given as a percentage of the total Cd present, in Table 113.

Table 113 : Percentage of Cd associated with different fractions

a) Roots

	Percentage Total Cd in Roots			
	Midger		Hallen	
Fraction	Test	Control	Test	Control
Soluble	16	11	37	9
Exch.	23	11	14	18
Carbonate	18	22	12	9
Organic	36	11	33	27
Reducible	3	22	2	9
Residual	5	22	2	27

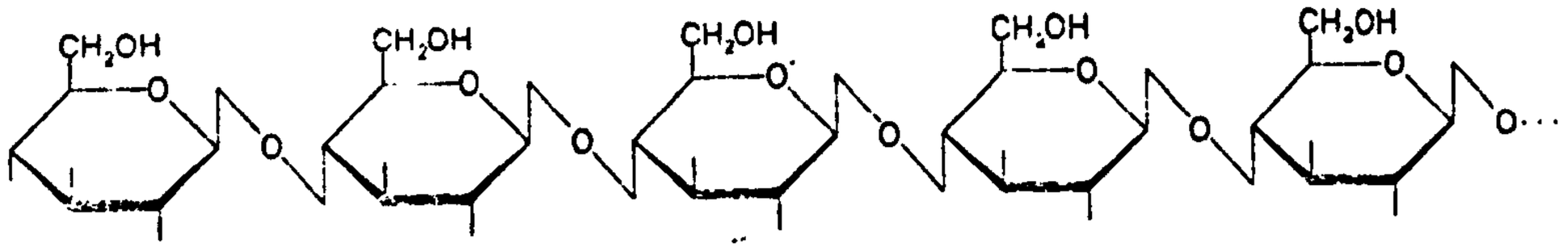
b) Shoots

	Percentage Total Cd in Shoots			
	Midger		Hallen	
Fraction	Test	Control	Test	Control
Soluble	6	17	14	20
Exch.	17	17	16	10
Carbonate	6	17	5	10
Organic	26	17	34	10
Reducible	2	17	5	10
Residual	43	17	27	40

Much of the Cd present in both populations is associated with the cell wall components, (organic - residual fractions) - pectin polymers, cellulose, hemicelluloses or lignin (339).

Cellulose (Figure 107) is insoluble in water, alcohols, ethers, chloroform, phenol, acetone, benzol, dilute alkalis, and in most organic

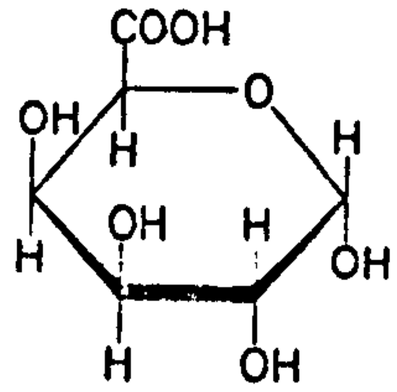
Figure 107



acids (312). The β-1-4 linkages of cellulose are highly resistant to acid hydrolysis; strong mineral acid is required to produce D-glucose (339,340) i.e. the extractants of the organic (H_2O_2/HNO_3) and residual (HF/HNO_3) fractions. The binding of the metal ions with cellulose is via the hydroxyl groups (30).

The pectins (or pectic substances), found in the primary cell walls and intercellular layers of land plants, are a group of complex plant polysaccharides in which D-galacturonic acid is the principal constituent :-

Figure 108 : Galacturonic acid



The D-galacturonic acid units combine with β-1-4 linkages to give polygalacturonic acid chains (341). Pectic substances, in general, are known to bind cations and have a cation exchange capacity (342). Metal chelation is via both carboxyl and hydroxyl groups (341,342,343). Interactions of an electrostatic nature allow some ions to retain the inner hydration shell, whereas inner sphere binding through the carboxylate groups produces immobilisation of some ions (343). Proto-pectates (i.e. the parent pectic substance (344)) can be hydrolysed free from the cellulose of the cell wall and converted into soluble 'pectin' by heating with dilute acids (345). Thus, metal release from the pectic substances begins at the carbonate fraction (i.e. pH 5 NaOAc/HOAc) and

is completed at the organic fraction (HNO_3/H_2O_2 and 85°C).

The majority of hemicelluloses are relatively small molecules consisting of between 50 and 200 monosaccharide residues with D-xylans being the most common of the hemicelluloses. The backbone of the molecule is an essentially linear chain of (1 - 4)-linked ß-D-xylopyranosyl residues. Other groups of hemicelluloses include L-arabino-D-xylans, D-mannans, and L-arabino-D-galactans (346). Metal complexation is probably again via-OH and -COOH groups. The hemicelluloses are more readily hydrolysed by dilute acid and even more easily dissolved in alkali, than cellulose (310). The carbonate (NaOAc/HOAc) and organic (H_2O_2, HNO_3) extractant will cause the breakdown of hemicelluloses present within the sample.

Lignin is a very complex three dimensional polymer of variable composition whose basic units include sugars, phenolic substances aromatic amino acids and alcohols (312,347). Chelation can therefore take place at any number of sites. Hydrofluoric acid rapidly converts cellulose and the associated carbohydrates into the corresponding sugar anhydrides, which are readily soluble in water, leaving lignin as an insoluble residue (312). The extractants of the residual fraction (HNO_3/HF and 98°C) are sufficient even to cause breakdown of the lignin.

The association of Cd with these components supports the evidence for the importance of the cell wall in metal ion inactivation (27). Because the extractants employed in the Tessier scheme are not specific for the plant components present; the scheme provides only a limited indication of the metal-plant fraction association. The scheme does, however, indicate that not only is the metal located in the cell wall, but is also present with other plant components and as the free ion. The cell wall apparently has a major role to play in metal inactivation which may help to explain differences in the metal concentration of the

roots and shoots. A cell wall complexing system suggests a finite capacity for absorbing metal (27). Some workers have proposed that phytotoxicity may occur when the capacity of root cell walls and other inert structures to absorb or accumulate Cd is exceeded (44). Other workers have concluded that the tolerance of plants to heavy metals could not be accounted for solely in terms of the properties of the cell wall compartment (31). In the present work, the results indicate that Cd was associated with plant components - possibly protein or organic acids (exchangeable and carbonate fractions) - other than the cell wall. No chlorosis or root damage was observed in either of the populations observed.

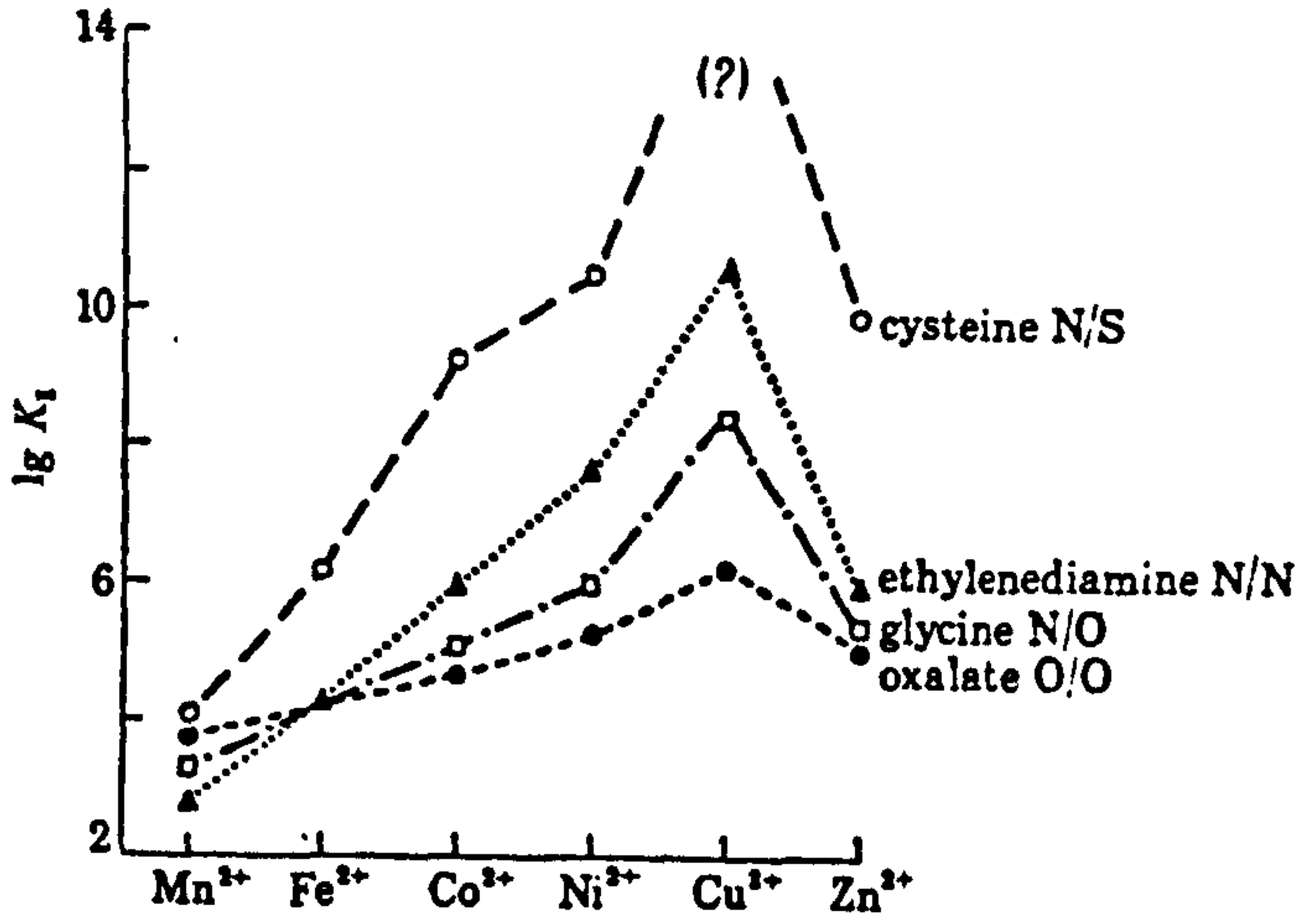
Girling and Peterson (157) suggested that Cd is probably transported as Cd^{2+}, but also found an association between protein and Cd. While Chino and Baba (23) proposed that Zn and Cd are transported as organometal complexes with citric acid or some other organic acid.

Hydrogen bonding between the central hydroxyl and carboxyl groups stabilises the planar conformation of citric acid. In chelates the cation lies close to the position of the H atom facilitating bidentate action of ligand and metal ion. Citrate is considered to be transported through cell membranes in the form HL^{2-}. It is speculated that metal citrate complexes can be transported in the same way. The ligand has only O atoms as donor atoms and therefore selectivity of the ligand for metal cations is poor (for example Figure 109).

Such organic acids are water-soluble and generally chemically stable (349).

In accordance with its Class B characteristics Cd has an affinity for SH groups (31,350) and therefore for proteins of the metallothionein type (31). The two strong metal binding residues in metallothioneins are sulphydryl (cysteine) and amine (lysine) groups. Since the number of

Figure 109 : The stability constants of different ligands in complexes of some trace elements (348)



sulphydryl groups is greater than the number of amines, the coordination environments around the metal ions are likely to be anionic, favouring the formation of a polynuclear arrangement. The role of the lysine residues in binding such metal ions is uncertain. At neutral pH, the lysine groups are probably not extensively coordinated. For each metal ion bound, three thiolate groups participate in coordination, some serving in a metal bridging capacity (351). Neutral salts often increase the solubility of neutral proteins and nearly all proteins dissolve in acid or alkaline solutions (352), (i.e. exchangeable - $MgCl_2$ - to organic extractants).

The Midger plants were found to contain 57% of the total root Cd in the first three fractions (44% for the control). For the Hallen plants

the value was 67% (36% for the control). Similarly for the shoots, the Hallen tests plants contained a greater proportion (35%) in these fractions when compared with the Midger plants (29%). The controls showed the reverse i.e. Hallen 40% and Midger 51%. Thus for the Hallen test plants there is a greater Cd association with the ionic/protein/organic acid fraction. The Cd tolerance of the Hallen population may be related to the presence of higher organic acid levels and/or a metallothionein-like protein, stimulated by the presence of Cd. A more specific analytical scheme is required to establish the metal-plant fraction association beyond doubt.

H. lanatus (Hallen) shoots by Quick scheme

Table 63 shows the levels of Zn, Cd, Pb and Cu in Hallen, H. lanatus shoots fractionated by the "Quick" scheme. The shoots were found to contain Zn, Pb and Cu, but not at excessively high levels, again demonstrating the possibility of restricted translocation to the shoots.

The percentage of total metal found in each fraction is given in Table 114.

Table 114 : Percentage of total metal associated with different fractions

	Percentage of total metal		
Fraction	Zn	Pb	Cu
Exch./Carb.	89	28	17
Reducible	0.4	13	10
Organic	-	20	-
Residual	11	40	73

Because the exchangeable and carbonate fractions have been combined by using a more acid (i.e: citric) extractant, these fractions together now mainly represent the soluble proteins, organic acids, pigments, pectic substances and hemicelluloses. The remaining plant components - cellulose and lignin - are found in the reducible-residual fractions. Cellulose is slowly attacked by alkali (308) (organic fraction extractant), and has been classified according to solubility criteria.

1) α -cellulose - the most stable portion of cellulose is defined on the basis of its insolubility in 17.5% aqueous NaOH. It is not a definite chemical entity, but a convenient empirically defined fraction of the cellulose. The molecular weight is $\sim$ > 15000 (312).

2) β -cellulose - is soluble in 17.5% aqueous NaOH but insoluble in dilute acid. The molecular weight is between 15,000 and 3000 (308,312).

3) γ -cellulose - is soluble in 17.5% aqueous NaOH and dilute acid. The molecular weight is < 3000.(308,312).

No Cu or Zn was found in the NaOH extracted ($\sim$ cellulose) fraction, while 20% of the Pb was found here.

Comparatively few soluble metal complexes from plants have been established. Complexes which have been identified include Cu bound to proteins, polypeptides and amino acids; and Zn as the aqua cation, as amino acid compounds and associated with pectates (342). Immobilisation of Cu and Zn may take place in cell walls as part of the mechanism of tolerance in some species (31). The 'normal' range of Zn in plants is 25 to 150 μg Zn/g dry matter (normal phytotoxic content > 400 μg Zn/g) and of Cu is 6-15 μg Cu/g dry matter (phytotoxic content > 20 μg Cu/g). Thus, from the Cu level found in the shoots, some Cu association with the cell wall fractions would be expected - 73% was located in the residual phase, compared with only 11% of the Zn. For Pb - thought to

be non-essential - 60% was associated with the organic-reducible fractions. Approximately 90% of the Zn and 27% of the Cu was located in the exchangeable/carbonate and reducible fractions. At higher Zn or Cu concentrations larger values of association with the cellulose fraction may be expected.

Clearly a more specific scheme is required to identify more closely the metal-plant component associations (such as the Farago speciation scheme, page 242).

Fractionation of Cu peat

Table 65 shows the results obtained when a Cu-peat sample was fractionated using an ethanol reflux and decreasing pH.

Boiling ethanol is often used in plant extraction procedures, being regarded as a good all-purpose solvent for preliminary extraction. For green tissue the success of extraction with alcohol is directly related to the extent chlorophyll is removed into the solvent. When the tissue debris is free of green colour, it can be assumed that all low molecular weight compounds have been extracted. Such compounds include phenolic acids bound as glycosides, the tricarboxylic cycle acids, non-protein amino acids, free sugars and lipids. The ethanol extract can then be further fractionated using solvents of varying polarity (349). From Table 65 approximately 28% of the Cu was extracted by the ethanol reflux. Very little Cu ($\sim$ 1%) was extracted by a second reflux with DDW. As the pH decreased the Cu concentration was found to increase. Much of the Cu extracted was present as organically bound metal - 47% of ethanol extracted Cu and 67% of the DDW extracted Cu. At pH 7, 20% of the 'ethanol' Cu and 4% of the 'DDW' Cu were labile. A further 7% of the 'ethanol' Cu was labile at pH 5 i.e. desorbed from colloids. A voltammogram (DPASV-HMDE) of the ethanol extracted Cu at pH 3 is given

in Figure 69.

The residue, remaining after both the ethanol and DDW refluxes, contained 71% of the total Cu extracted. More severe extraction conditions are required to leach the higher molecular weight compounds present (e.g. cellulose, lignin, humic acid).

Fractionation of Cd and Cu peat samples by Farago scheme

Tables 66a and 67a illustrate the results obtained from the fractionation of Cd and Cu peat by a modified version of the Farago Scheme.

The Farago scheme permits the identification of metal-plant component associations, but suffers from the same disadvantage encountered with other fractionation procedures, in that chemical changes may occur which can lead to incorrect conclusions (342). However, some idea of the metal compartmentalisation within the plant can be achieved.

After the ethanol and DDW refluxes, the residue was treated with papain at pH 7 and in the presence of chloramphenicol. The substrate specificity exhibited by enzymes (339) was used here to obtain the protein/amino acid fraction. Papain is a proteolytic enzyme (353), which utilises the thiol group of cysteine as the nucleophilic reagent for the hydrolysis (350) and has a pH optimum of approximately pH 7 for most substrates (354,355). Solutions of chloramphenicol in water are faintly acidic (pH 5.5) (356). The antibiotic inhibits many gram-positive and gram-negative cocci and bacilli (356); by interfering with protein synthesis (357).

Because enzymes are such efficient catalysts, very low concentrations suffice to allow a reaction to proceed at a measurable rate: concentrations of 10^{-8} to 10^{-10} M are typical. Substrate concentrations are usually greater than 10^{-6} M. The simplest possible enzyme mechanism is the

conversion of a single substrate to a single product. Many enzyme reactions have two substrates and two products. The rate of catalysis is generally decreased, in the presence of substances structurally similar to the substrate (358). The degradation of lignin has recently been carried out using an enzyme isolated from the White Rot fungus Phanerochaete chrysosporium. Lignin is considered difficult to study because, in contrast to cellulose, it lacks steric regularity and monomer chirality. Lignins are very variable, with softwoods and hardwoods, having distinctive monomer proportions; grass lignins contain in addition phenolic acids bound to the polymer by ester bonds (347). Future fractionation schemes may be able to incorporate such enzymes, to ensure the breakdown of known plant components.

The insoluble pectates may be freed by treating with ammonium oxalate, in as much as the ammonium pectate is soluble (345).

Isolation of lignin can be conveniently divided into two classes :-

1) removal by hydrolysis,of the cellulose and other components, leaving lignin as an insoluble residue, or,

2) removal of lignin from the cellulose and other substances with which it is associated.

Method 1 can be carried out using fuming hydrochloric acid. Hydrochloric acid (of 40 to 42% strength) readily dissolves cellulose, leaving lignin as an insoluble residue, which can be acid digested with nitric acid (312).

Method 2 is achieved by dissolving the lignin in aqueous potassium hydroxide, leaving the cellulose as an insoluble residue. Lignin is fully degraded by the action of boiling KOH (345).

From Table 66a, Cd was found in all fractions - the majority being associated with the cell wall constituents, especially lignin and cellulose. Very little (~1%) was associated with the low molecular weight materials. The remainder (~12%) was found in the protein/amino

acid fraction. Much of the Cd was firmly bound within the peat, requiring for its removal the more severe extraction conditions of the later stages of the scheme.

Copper was also found in all fractions (see Table 67a) and again was associated to a large extent with the cell wall constituents, but mainly with the insoluble pectates and hemicelluloses. A higher proportion of Cu was found in the low molecular weight and protein/amino acid fractions. The Cu was extracted more readily than the Cd, possibly as a result of the greater degree of Class B character attributed to Cu (31) and hence the greater affinity for S and N centres found in amino acids,proteins and pigments.

The summed totals and acid digest totals agree well for both Cu and Cd. The scheme therefore provides an efficient method of extraction.

Fractionation of plant samples

Here, the ethanol extract was further fractionated using DDW, ether, chloroform/n-pentanol and acetone.

The extract was initially mixed with DDW and ether - separation of the pigments is achieved by partition between the polar and non-polar solvents (359). The DDW extract was mixed with chloroform/n-pentanol. Proteins soluble in the organic solvent (i.e. proteins containing a high proportion of valine, leucine, isoleucine, phenylalanine, tryptophan or methionine (360)) are partitioned in the chloroform fraction, leaving the more polar plant components such as amino acids and organic acids in the DDW phase. The DDW fraction was finally shaken with acetone. The pectins are obtained from the solution by precipitation with acetone (344), after undergoing an aldol condensation reaction (361). The water layer contains low molecular weight materials such as tricarboxylic cycle acids and amino acids.

SRM 718 Orchard leaves. Tables 66b,67band 68

The Zn was associated mainly (59%) with the protein/amino acid fraction, the remainder being located in the cell wall components - especially cellulose and lignin (25%). The summed total of the fractions was slightly greater than the NBS value of 25.0 µg/g i.e. obviously some contamination had occurred.

Due to its enzymic role the Zn-protein association is to be expected and since the Zn level in the leaves is not excessive the low Zn concentration found in the cell wall fractions is understandable. Loss of sample would explain the low acid digest value. As no precipitate was formed on the addition of acetone to the water extract, it can be concluded that the level of soluble pectates was very low.

No Cd was determined in the fractions; the NBS Cd value is 0.11 µg/g and the acid digest 'total' value was similar (0.13 µg/g). Dilution of this low Cd content (by the extractants used in the scheme) prevented the Cd determination by the AAS method of analysis. Determination by the more sensitive technique of DPASV was precluded, due to the organic nature of the sample.

The Pb showed a similar pattern of distribution to that of Zn - but was associated with the cell wall fractions to a greater extent - i.e. 58% of the Pb was found in fractions Y and Z, the Pb showing greater association with cellulose, and Zn with lignin and the hemicelluloses. The presence of Pb in the cell wall fractions would be expected, since Pb is regarded as a non-essential element. The Pb association with the protein/amino acid fraction may indicate a tolerance mechanism of the metallothionein type or may be due to Pb binding to the SH groups present in the "normal" plant proteins. Sulphur, absorbed mainly in the form of sulphate ions, is reduced in plants and incorporated in organic compounds and is a constituent of the amino acids cystine, cysteine and

methionine, and hence of the proteins containing these amino acids. Thiamin, biotin, and coenzyme A are sulphur-containing low molecular weight coenzymes essential for metabolism when attached to appropriate apoenzymes (proteins) which require these coenzymes or prosthetic groups for catalytic activity. The ferredoxins, non-heme iron proteins involved in photosynthesis and other electron transfers contain sulphur in amounts equivalent to the iron present. Proteins are the compounds in which most of the nitrogen and the majority of the sulphur contained in plant tissues are incorporated. When in ample supply, the absorption of sulphate may be faster than the assimilation of its sulphur atoms into organic compounds. An appreciable fraction of the total sulphur in plants may therefore be in the form of sulphate (362).

The Cu was found in a greater number of fractions compared with either Pb or Zn. The majority (~ 73%) was located in the protein/amino acid fraction. Copper was also found in the soluble protein fractions. A very small proportion (0.5%) was located with the polar low molecular weight materials e.g. amino acids, while 2% was found in the poly-saccharide fraction. The remainder (~16%) was located with the cell wall components.

Copper is contained in several essential enzymes, particularly those reducing oxygen to water e.g. ascorbate oxidase and laccase. The Cu metalloprotein, plastocyanin is involved in the electron transport chain in photosynthesis. There is a strong association between Cu and nitrogen content of leaves (145). Amino acids, which are generally present in relatively much higher concentrations than Cu^{2+} in the xylem sap form very strong complexes with Cu^{2+} (363,64). The association of Cu with the protein/amino acid fraction is, therefore, not surprising.

Hallen H. lanatus:Tables 69 to 72

High Zn levels were found in the roots and shoots of this sample; i.e. > 1100 µg/g which is a factor of nearly 3 above the normal phytotoxic level (40).

The Zn content of the roots was much higher than that of the shoots, again indicative of restrictive translocation to the shoots. Most of the root Zn was present either in the protein/amino acid (~53%) or in cell wall fractions (~43%). The remainder was present in the pigment (~1%) and low molecular weight (~2%) fractions.

The vast majority of the shoot Zn was located in the cell wall (~95%) - especially cellulose (~67%). The remainder was present with the proteins/amino acids (~5%) and pigments (~1%).

The cell wall components for both roots and shoots were thus found to play a major role in Zn compartmentalisation. Root proteins were also of importance - indicative, possibly, of a metallothionein-like role. The presence of Zn in other fractions may be due to the saturation of the root cell walls and proteins with metals. Alternatively it may be due to the contamination of one fraction with another through incomplete separation e.g. ether extracted lipids present in the pigment fraction, water soluble proteins (i.e. those containing a high proportion of aspartic acid, glutamic acid, lysine, arginine, or histidine (360)) in the low molecular weight fraction.

Much less Cd than Zn was found in the plant fractions. Again the Cd level was higher in the roots than the shoots. Most of the root Cd was present in the soluble protein and protein/amino acid fractions - ~67%. However some Cd was also found in the polar low molecular weight (~25%) and ∝-cellulose/lignin (~8%) fractions. The shoot Cd was equally distributed between the pigment, soluble pectate, soluble protein and protein/amino acid fractions.

For Cd (regarded as a non-essential element) the proteins and cell wall components play a major role. The protein association may be due to a metallothionein-like effect, or may be due to substitution of Cd^{2+} for Zn^{2+} in Zn enzymes (7). Since there is a ready supply of Zn the latter is unlikely. The presence of Cd in the polar low molecular weight fraction can be attributed to Cd-amino acid, or Cd-organic acid complexation.

A high level of Pb was found ÷ the roots containing a factor of 9 greater than the Pb content of the shoots. The Pb was located in the protein/amino acid fraction (~ 25% of the root Pb and 40% of the shoot Pb). The remaining Pb was located in the cell walls of both roots and shoots - mainly in the ∝-cellulose/lignin fraction (~38% root, ~ 40% shoot).

For Pb also, protein and the cell walls are sites of accumulation.

The Cu content of the Hallen plants exceeded the normal phytotoxic concentration of 20 µg/g (40). The roots and shoots contained very similar Cu levels, but differed in the pattern of distribution. The root Cu was located in the soluble protein (~35%), and cell wall fractions. The shoot Cu was located in the protein/amino acid fraction (~51%), as well as the cell wall fractions. For Cu, as for the other metals, the proteins and cell wall components are important.

Callington plant samples : Tables 73 to 75

A high level of Zn was found in the grass (Agrostis tenuis) sample, but did not exceed 400 µg/g (usual phytotoxic concentration (40)). The Zn was distributed among the low molecular weight materials e.g. monosaccharides (~ 8%), the soluble protein (~11%), proteins/amino acids (~16%), polysaccharides e.g. starch (~6%) and cell wall components (~60%).

The association of Zn with the low molecular weight materials may be

due to organic acid chelation, as suggested by Mathys (146). Clearly this finding requires further investigation.

The Zn moss level was within the normal plant Zn range of 25 to 150 µg/g (40), and was mainly associated with the cell wall components (~97%), the remainder was found in the protein/amino acid fraction. Mosses have evolved efficient uptake mechanisms for absorbing metals and other nutrients from their environment. The greater part of the metal content of mosses is not taken up and located within the cells but is accumulated extracellularly in two ways, i.e. via ion exchange or particulate trapping.

a) Ion exchange

The cell walls of mosses possess ion exchange sites onto which metal ions bind in a way comparable to the binding of ions onto synthetic ion exchange resins. If a moss (or a lichen) is placed in a solution of a metal salt, ion exchange takes place in a matter of minutes and does not require the expenditure of metabolic energy.

b) Particulate trapping

The surface of moss gametophores provides innumerable crevices for the entrapment of tiny particles. The cell wall-metal association exhibited by Rhyatidiadelphus squarrosus is therefore not surprising. R. squarrosus, watered with a lead acetate solution, was found to contain some of the Pb in electron dense vesicles. The vesicles are thought to be part of a detoxification system (364).

Very little Cd was found in the grass sample - which was associated with proteins and amino acids. No Cd was found in the moss.sample.

A high Pb content was found in the grass sample - ~ 4% was located in the protein/amino acid fraction ,~6% in the low molecular weight fraction and the remainder in association with the cell wall components -

especially ∝-cellulose/lignin sub-fraction (53%). For the moss, the Pb was also associated with the protein/amino acid (~19%) and cell wall (especially ∝-cellulose/lignin ~43%).

The Cu content of both the moss and grass exceeded the normal phytotoxic content (moss by a factor of 4, the grass by a factor of 14). For A. tenuis, the Cu was found throughout nearly all of the fractions - ~21% protein/amino acids, ~ 3% soluble protein, ~6% low molecular weight materials, ~ 1% pigments, ~ 5% polysaccharides and ~ 1% polar low molecular weight materials. However, the remaining Cu (the majority) was associated with the cell wall fraction (~63%).

The presence of the Cu in nearly all fractions (only soluble pectates, fraction F and lignin fraction Z' did not contain Cu) may result from saturation of the cell wall and protein sinks.

The moss Cu was also widely distributed but was not found in fractions S (soluble protein), V (low molecular weight materials), F(soluble pectates), G (polar low molecular weight materials) or Z' (lignin). Much of the Cu was associated with the protein/amino acids (~24%) and cell wall components (~61%). The remainder was located with the pigments (~2%), soluble protein (~4%) and polysaccharides (~9%),

Luckett plant samples : Tables 76 to 79

The Zn content of both grass and moss samples was below the normal phytotoxic content (400 µg/g (40)), but higher than the normal Zn plant content range (25 to 150 µg/g) - especially the A.canina sample. The Zn was found in only four fractions for both samples.

The grass Zn was found in the protein/amino acid (~12%) fraction and in association with ∝ -cellulose and lignin (~88%) - cellulose in paticular (~ 40%).

The moss Zn was located in the ∝ -cellulose/lignin fraction (~50%),

α-cellulose fraction (~49%), lignin (0.5%) and protopectate (0.5%) fractions. The results demonstrate the importance of the cell wall in mosses. Zinc is a borderline metal, which normally exhibits a significant degree of class A character (364), as illustrated here by an affinity for O coordination. The cell wall is a major site of Zn accumulation in both samples.

Cadmium was found in both samples, but was well below the normal phytotoxic content of 100 µg/g (40). The grass Cd was located entirely in the protein/amino acid fraction - demonstrating the class B character of Cd and its affinity for N/S centres (31). The protein/amino acid (~12%), soluble protein (~18%) and polar low molecular weight materials (~35%) - such as amino acids - fractions were also of importance in the moss sample. The remainder was located with the cell wall constituents.

High Pb levels (well over the normal Pb range of 2-14 µg/g (40)) were present in both the moss and the grass - in particular the latter. In both cases, Pb was mainly associated with the cell wall components. For the grass, Pb was also present with the soluble protein (~ 3%) and polysaccharides (~9%). A slightly higher proportion of the moss Pb was associated with proteins (~5% soluble protein and ~4% protein/amino acids). The α-cellulose/lignin fraction contained most of the moss Pb (~64%).

High Cu levels were also found in the Luckett samples (a factor of 12 greater than the normal phytotoxic level was found in the grass and a factor of 23 greater than the phytotoxic level in the moss). Much of the Cu for both samples was located in the cell wall fractions. In A. canina Cu was also present with the soluble protein (~3%), protein/amino acids (~11%) and polysaccharides (~ 5%). For C. purpureus Cu was present in nearly all the fractions, being absent from fraction S (soluble protein), F (soluble pectates) and Z' (lignin). Very little Cu

in the moss was associated with protein - ~1% soluble protein, ~ 3% protein/amino acids and 0.4% polar low molecular weight materials. A small percentage (~1%) was located with the low molecular weight materials, and ~ 5% was with the polysaccharide fraction. The cellulose fraction contained most of the Cu (39%). Thus, in moss in particular, the cell wall constituents were major sinks for Cu.

Goginan, Merlin, Parys Cd samples : Tables 80 and 81

Cd was readily taken up by all three populations - high Cd levels (greatly exceeding the normal phytotoxic content of 100 µg/g (40)) were found in these plants. The Cd concentration was much greater in the root than the shoot, again indicative of restricted transport. The three populations although reported to be tolerant -

i) Goginan for neutral and acidic Pb and Zn,

ii) Merlin for neutral and calcareous Pb and Zn, and

iii) Parys for neutral and acidic Cu (127)

have not been reported to be tolerant to Cd. Despite this, no obvious ill effects were noticed in any of the plants studied. Parys plants were found to contain the highest total Cd concentration and Merlin the least, but the highest shoot Cd level was found in Merlin. Goginan plants, therefore, showed the greatest tolerance to the metal in terms of restricted translocation and Merlin in terms of restricted uptake. The greater degree of Cd tolerance exhibited by these two populations may be related to their resistance to Zn toxicity, in that Zn^{2+} and Cd^{2+} have very similar chemical characters (7). However, the Cu-tolerant Parys plants, although taking up a high Cd concentration, also exhibited restricted Cd transport and did not exhibit any obvious toxicity symptoms. The two A. tenuis populations (Parys and Goginan) may possess similar mechanisms of tolerance, whilst showing varying degrees of tolerance, due

to different efficiencies of operation of the mechanism, i.e. which may involve the efficiency of energy expenditure required to maintain metal tolerance (359). A similar effect was noted by Brown and Martin (140) for two populations of Holcus lanatus.

For all three of the populations studied, the protein/amino acid fraction of the roots was the major site of metal accumulation (Goginan ~ 61%, Merlin 53%, Parys 53%). The control plants contained very little Cd (none was found in the Parys control plants), but the Cd found was also located in the protein/amino acid fraction.

The root Cd was also associated with the soluble protein fractions (Goginan ~ 1%, Merlin ~ 17% and Parys ~3%), and the low molecular weight (Goginan ~ 13%, Merlin ~ 6%, Parys ~ 15%) and polar low molecular weight (Goginan ~ 7%, Merlin ~ 8% and Parys ~ 6%) fractions. The remainder was present with the cell wall components (Goginan ~ 18%, Merlin ~ 15% and Parys ~ 23%).

A greater proportion of the shoot Cd was associated with the cell wall fractions (Goginan ~ 60%, Merlin ~ 56% and Parys ~ 80%). The protein/amino acid fraction contained ~ 36% of the Goginan Cd, 35% of the Merlin Cd and 13% of the Parys Cd. The remaining shoot Cd was found in the low molecular weight (Goginan 1%, Merlin ~ 6%), soluble protein Goginan ~2%, Parys ~4%) and polar low molecular weight (Goginan 1%, Merlin ~ 3%, Parys ~ 3%) fractions (Table 115). The results obtained are summarised in Table 115 .

Obviously, for the three populations, proteins play an important role in the accumulation of Cd, indicating the possibility of a metallothionein-type protein, as reported by other workers for Cd treated plants (155,156,158,365). Since the root cell walls contained a much lower proportion of Cd than was found associated with the protein, the protein accumulation appears to be the major tolerance mechanism. The

Table 115 : Summary of the fractionation of Goginan, Merlin and Parys grown in Cd amended culture solution

Fraction	Percentage of Total Cd					
	Goginan		Merlin		Parys	
	Root	Shoot	Root	Shoot	Root	Shoot
Proteins/amino acids	62.8	37.9	70.1	35.0	55.2	17.0
Polar/low M(r) materials	19.8	2.0	14.6	9.3	21.4	2.9
Cell wall components	17.5	60.1	15.3	55.6	22.8	80.1
Polysaccharides	-	-	-	-	0.6	-

most tolerant population - Goginan - was found to contain the highest proportion of root as well as shoot Cd in the protein fraction, while the Merlin population, which exhibited reduced Cd uptake, was also found to contain a high proportion of shoot Cd in this fraction. It is possible that saturation of the protein binding sites would lead to Cd complexation by the cell wall components. The Parys population exhibited the lowest proportion of Cd-protein and highest Cd-cell wall association, i.e. the possibility of the reduced efficiency in the use of a protein-metal tolerance mechanism.

Figure 71a demonstrates the problem of metal determination in a plant extract using an RDE. The absence of any peaks in the sample spike scans is probably due to the organic interference noted earlier.

Goginan, Merlin, Parys Pb samples : Tables 82, 83 and 84

Lead was readily taken up by the three populations. Very high levels were found in the roots but the levels were much lower in the shoots (i.e. restricted transport to the shoots) of Goginan and Merlin - the two Pb tolerant populations. The Parys plants were found to accumulate the most

Pb and contained a very high shoot-Pb concentration. Much of the Parys root Pb (48%) was found with the protein/amino acids. A small percentage was found with the soluble protein (~0.1%) and polar low molecular weight (~ 2%) fractions. The remainder was associated with the cell wall components (~50%). For Goginan and Merlin the root Pb was mainly present in the cell walls (Goginan ~ 84% , Merlin ~ 73%) - the cellulose/ lignin fraction in particular (Goginan ~ 43%, Merlin ~ 31%). The protein/amino acid fraction contained ~ 9% of the Goginan root Pb and ~ 21% of the Merlin root Pb. A small percentage of Merlin root Pb was located in the soluble protein (~ 1%) and polar low molecular weight (~ 1%) fractions. The remaining Pb was associated with the polysaccharide fraction (Goginan ~ 6%, Merlin ~ 5%). For the control plants Pb was found at low levels and in only 3 fractions (hemicelluloses Goginan, protein/amino acids Merlin, polar low molecular weight fraction Parys).

The Goginan shoot Pb was associated entirely with the cell wall components (~ 85%) (excluding the pectins) and polysaccharides (~15%). The Merlin shoot Pb was found mainly with the cell wall components (~93%). The remainder was present in the protein/amino acid (~ 6%), polar low molecular weight (~ 1%) and soluble protein (~ 1%) fractions. For Parys, ~ 2% was in the protein/amino acid and ~ 11% in polysaccharide fractions. The remaining Pb was in the cell wall fractions including the pectates, as illustrated in Table 116.

Pb, therefore, shows a different distribution to that of Cd, the protein fraction is not as important for Pb as it is for Cd. Instead the cell wall - especially cellulose/lignin - is a major site of metal accumulation. For the Parys species the pectate fraction was also of importance - possibly due to saturation of all other sites. The protein-Pb association found in this population may not be as a result of a tolerance mechanism, since high Pb levels were taken up and transported

Table 116 : Summary of the fractionation of Goginan, Merlin and Parys grown in Pb amended culture solution

Fraction	Percentage of Total Pb					
	Goginan		Merlin		Parys	
	Root	Shoot	Root	Shoot	Root	Shoot
Proteins/amino acids	9.3	-	21.7	6.6	48.3	1.8
Polar/low M(r) materials	-	-	0.9	0.5	1.5	-
Cell wall components	84.4	85.2	72.6	93.1	50.2	87.2
Polysaccharides	6.3	14.9	4.9	-	-	10.9

to the shoots - the association may, therefore, be due to Pb 'normal' plant protein binding, such as blockage of enzyme-SH groups (7), as mentioned earlier.

Tables 83a and b show the Zn distribution found in Goginan, Merlin and Parys plants, hydroponically grown in Pb amended culture solution. Few studies have been carried out to investigate interactions in uptake of heavy metals in higher plants, but where such studies have been carried out positive effects have been found (139,342,365,39,57).

Zn was readily taken up. High Zn concentrations (exceeding the normal phytotoxic content) were found in the three populations. Restricted translocation to the shoots was again apparent. The lower Zn concentrations were exhibited by the Zn tolerant Goginan and Merlin. The Zn levels found in the Pb treated plants were greater than the high Zn levels found in the control plants (Tables 85a and b). Coughtrey and Martin (139) noted that Zn tended to be decreased by Pb, in a study of two populations of Holcus lanatus. Other workers have observed inhibition of Zn^{2+} absorption in a Hoagland's nutrient medium containing Pb (57). However, growth stimulation noted when Pb has been present in

the growth medium is thought to result from ion(s) other than Pb being made available (57), possibly a lead-phosphate interaction allowing greater Zn availability.

The Zn distribution differed from that of Pb. The polar low molecular weight fraction of the roots contained much of the A. tenuis populations' Zn (Goginan ~ 64%, and Parys ~ 44%, compared with ~ 9% for Merlin). The Merlin root Zn was associated mainly with soluble protein (~ 46% Merlin, compared with ~ 15% for Goginan and ~ 27% for Parys). The cell wall components contained ~ 21% of the Goginan root Zn, ~ 27% of Merlin root Zn and ~ 21% of the Parys root Zn. A proportion of root Zn was also found in the pigment fraction for Merlin (~18%) and Parys (~ 8%).

The shoot Zn was found in a number of fractions - the cell wall components were important (~ 61% Goginan, ~ 51% Merlin and ~ 30% Parys), as was the soluble protein fraction (~ 25% Goginan, ~ 42% Merlin and ~ 36% Parys). A percentage (~ 14%) of the Goginan shoot Zn was also found in the pigment fraction, while the polar low molecular weight fraction also contained Zn (~ 4% Merlin, ~ 9% Parys). The remaining Merlin shoot Zn was associated with the polysaccharide fraction. The results are summarised in Table 117.

Table 117 : Summary of the fractionation of Goginan, Merlin and Parys plants grown in Pb amended culture solution - Zn content

	Percentage of Total Zn					
	Goginan		Merlin		Parys	
	Root	Shoot	Root	Shoot	Root	Shoot
Pigments	-	13.5	18.0	-	8.0	-
Proteins/amino acids	14.6	25.4	46.4	41.8	26.9	61.0
Polar/low M(r) materials	64.0	-	8.6	3.6	43.7	8.9
Cell wall components	21.4	61.0	27.0	50.3	21.3	30.0
Polysaccharides	-	-	-	4.3	-	-

The Zn distribution thus differed from that determined for Pb (mainly cell walls) and Cd (mainly protein) in that the Zn was found associated with protein, cell wall components and with polar low molecular weight materials. The latter association may be due to Zn-amino acid or Zn-tricarboxylic acid complexation. Increased levels of organic acids have been found in some Zn tolerant plants (143,142) and a tolerance mechanism involving organic acid-metal complexation has been proposed (146). The A. tenuis and F. rubra populations differed in their association with this fraction - soluble protein was found to be a more important site of Zn accumulation in the Merlin (F. rubra) plants.

Table 84 (a & b) shows the distribution of Cu in Goginan, Merlin and Parys plants grown in Pb amended culture solution.

Copper was readily taken up by the Merlin and Parys plants - especially the Cu tolerant Parys population. The Goginan plants contained much less Cu, but the Cu concentrations in all the plants exceeded the normal phytotoxic content (a factor of 3 for Goginan, a factor of 14 for Merlin and a factor of 30 for Parys). Uptake to the shoots was restricted in the Merlin and Parys populations. But for Goginan the shoot concentration was higher than that of the root. Under normal conditions (i.e. not toxic or deficient) between 50 and 80% of the total Cu is associated with plastocyanin (304). The presence of Pb in the nutrient solution apparently causes an increased Cu uptake in the Merlin and Parys plants (compared with non-amended nutrient solution, Table 88), whilst for Goginan the Cu uptake was less than for the control population.

The Parys root Cu was present mainly in the soluble protein (~27%), low molecular weight (~33%) and protein/amino acid (~27%) fractions. Only ~ 10% was associated with α-cellulose/lignin, ~ 2% was in the polysaccharide fraction. The remainder was associated with the pigments.

For Merlin, the root Cu was associated with the cell wall fractions to a greater extent (36%), but was also present in the soluble protein (~10%), pigment (~ 20%) and low molecular weight (~34%) fractions. The Goginan root Cu content was found entirely in the low molecular weight fraction.

The shoot Cu for Parys was also located mainly in the soluble protein (~ 48%), low molecular weight (~ 31%) and protein/amino acid (~15%) fractions. The remainder was associated with the cell wall. The majority of the Merlin shoot Cu was located in the cell wall fractions (~ 86%). The soluble protein (~4%) and low molecular weight material (11%) contained the rest. For Goginan, the low molecular weight material (47%) was also important and the polysaccharide fraction contained much of the shoot Cu (~ 37%). The only other fraction found to contain Cu was the soluble proteins (Table 118).

Table 118 : Summary of the fractionation of Goginan, Merlin and Parys grown in Pb amended culture solution. Cu content

	Percentage of Total Cu					
	Goginan		Merlin		Parys	
Fraction	Root	Shoot	Root	Shoot	Root	Shoot
Pigments	-	-	20.3	-	1.9	-
Proteins/amino acids	-	15.8	9.5	3.6	53.2	62.9
Polar/low M(r) materials	100.0	47.4	34.2	10.9	33.4	30.6
Cell wall components	-	-	36.0	85.5	9.6	6.5
Polysaccharides	-	36.8	-	-	1.9	-

The proteins and low molecular weight materials tended to be the most important sites of Cu accumulation, indicating possible amino acid, and tricaboxylic acid complexes, as well as Cu-protein associations.

Cu-amino acid complexes have been proposed as part of the Cu transport process (31 & 56), and an intracellular pool of Cu-amino acid or similar species has been suggested (56). Cu-chelatin and Cu-metallothionein have also been isolated (154,160).

Table 85 shows the Zn distribution in Goginan, Merlin and Parys plants from non-amended culture solution.

High Zn levels were found in all three populations - exceeding the normal phytotoxic content - but lower than that found for the plants grown in the Pb amended solution. The Zn distribution differed between the "Pb plants" and the controls, the Zn-polar low molecular weight materials association being much less in the control plants.

The Parys root Zn was predominantly associated with the soluble protein fraction (~83%), the remainder was located with the cell wall components. The same pattern was ascertained for the other A. tenuis population (Goginan : ~ 69% soluble protein; ~ 31% cell wall fractions). The Merlin population showed a greater Zn association with the cell wall components (~ 83%) than the soluble proteins (~17%).

For the shoots, the Zn contained in the Parys sample was mostly located in the cell wall (93%), with the soluble protein fraction containing the remainder. For Merlin grass the cell wall contained ~ 80% and the soluble protein ~ 5%, the remainder being associated with the polysaccharide fraction. The Goginan plants (which accumulated the greatest quantity of Zn) showed a greater Zn association with the low molecular weight materials (~ 73%). The soluble protein content was only ~ 2%, and the cell wall components ~ 25%. A small percentage (~ 0.3%) was associated with the polar low molecular weight materials.

The Merlin plants, which accumulated the least Zn, were found to contain much of the Zn in the cell wall fractions, differing from the

plants grown in Pb amended solution, where much of the Zn was found in the protein fractions. However in the "Pb plants", more than twice the quantity of the Zn had been accumulated.

The Goginan plants accumulated similar Zn levels in the controls and "Pb plants". The low molecular weight fractions were important to both systems. In the absence of Pb, the Parys plants accumulated less Zn. The Zn present was associated with the protein fractions to a greater extent and with the low molecular weight materials to a lesser extent, than was found for the "Pb plants".

It appears that for Merlin at lower levels of Zn accumulation, the cell walls were the predominant sink, while at intermediate levels the proteins may play a more important role. For Parys and Goginan species the low molecular weight materials tend to be more important at higher Zn contents. A summary is given in Table 119.

Table 119 : Summary of the fractionation of Goginan, Merlin and Parys grown in non-amended culture solution. Zn content

	Percentage of Total Zn					
	Goginan		Merlin		Parys	
Fraction	Root	Shoot	Root	Shoot	Root	Shoot
Proteins/amino acids	68.7	2.3	17.2	5.3	82.8	7.0
Polar/low M(r) materials	-	73.1	-	-	-	-
Cell wall components	31.4	24.6	82.8	79.6	17.3	93.0
Polysaccharides	-	-	-	15.1	-	-

Table 86 shows the fractionation of Goginan, Merlin and Parys plants grown in Zn amended culture solution. Very high Zn levels were present in all three populations, as shown in Table 120.

Table 120 : Total Zn concentrations found in Goginan, Merlin and Parys populations grown in nutrient solutions amended with different metals

Zn concentration µg/g

TABLE NO.	GOGINAN	MERLIN	PARYS	TREATMENT
85	1583	608	1033	CONTROL
90	685	1441	2341	Cu
83	1461	1574	2684	Pb
86	3384	2327	2871	Zn

Although high Zn levels were found in the shoots, the concentrations were lower than those determined for the roots. The majority of the Parys Zn root was found in association with soluble protein (~41%), polar low molecular weight materials (~31%), and cell wall components (~17%). A small percentage (0.3%) was found with the polysaccharides and ~ 11% was found in the pigment fraction - this latter may be due to saturation of binding sites causing 'overflow' into other fractions. The pigment fraction was also important in the Merlin population (~39%), but little Zn (~ 2%) was associated with proteins. The cell wall contained ~ 29%, the low molecular weight materials ~ 9% and polar low molecular weight fraction ~ 21%. For Goginan, the major sites of Zn accumulation were the polar low molecular weight materials (~32%), and the cell walls (~ 55%), the remainder being located in the pigment (~5%), low molecular weight materials (~ 5%), soluble protein (~0.3%) and polysaccharide (~ 3%) fractions.

The Parys shoot Zn was in the soluble protein (~22%), polar low - molecular weight (~25%), protein/amino acid (~10%) and cell wall (~43%) fractions. The Merlin shoot Zn was found in the proteins (~13%), polar

low molecular weight materials (~10%), and cell walls (~77%). For Goginan, the polysaccharide fraction was a major site of accumulation (~30%), as were the cell walls (~52%). The soluble protein content was ~6%, protein/amino acid ~5% and polar low molecular weight materials ~7% (See Table 121).

Table 121 : Summary of the fractionation of Goginan, Merlin and Parys grown in Zn amended culture solution

	Percentage of Total Zn					
	Goginan		Merlin		Parys	
Fraction	Root	Shoot	Root	Shoot	Root	Shoot
Pigments	5.3	-	38.5	-	10.8	-
Proteins/amino acids	0.3	10.3	2.0	13.1	41.0	31.5
Polar/low M(r) materials	37.1	7.4	30.4	9.5	31.2	25.2
Cell wall components	54.8	51.9	29.2	77.2	16.7	43.3
Polysaccharides	2.5	30.3	-	-	0.3	-

At these high Zn levels, the polar low molecular weight materials are important for Parys and Goginan but to a lesser extent for Merlin. At this Zn level the protein-Zn association found in the Merlin "Pb plants" was not observed, instead there was a much greater association with the polar low molecular weight materials and pigments. The Zn-pigment association may be due to "overflow" as mentioned earlier, possibly resulting from competition for protein binding sites between Zn and Cu (Cu accumulation was greater in the "Zn plants" than the controls; Tables 87 and 88). It appears that the polar low molecular weight materials . become important sites for Zn accumulation in all three plant populations when the Zn concentration is high.

Table 87 shows the distribution of Cu in Goginan, Merlin and Parys plants grown in Zn amended culture solution.

Copper was taken up in all three populations to a greater extent than was found for the control plants while the shoot Cu concentrations were lower than those of the roots.

The Parys root Cu was located in the cell wall (~ 70%) and low molecular weight (~ 30%) fractions. For Merlin the Cu was mostly associated with the soluble protein (~ 62%) and low molecular weight (~ 29%) fractions, only ~ 9% was located in the cell wall. The Goginan root Cu was also mostly associated with the soluble protein (~ 40%) and low molecular weight (~ 42%) fractions, the remainder being found in the cell walls (~ 16%) and the polysaccharide (~2%) fractions. The shoot Cu showed similar distributions, again mainly being found in the soluble protein, low molecular weight or cell wall fractions, as demonstrated by Table 122.

Table 122 : Summary of the fractionation of Goginan, Merlin and Parys grown in Zn amended culture solution. Cu content

	Percentage of Total Cu					
	Goginan		Merlin		Parys	
Fraction	Root	Shoot	Root	Shoot	Root	Shoot
Proteins/amino acids	40.4	33.3	62.3	23.4	-	25.0
Polar/low M(r) materials	41.6	66.7	29.0	38.3	29.6	41.7
Cell wall components	16.1	-	8.7	38.3	70.4	33.3
Polysaccharides	1.9	-	-	-	-	-

Thus, under these conditions the Cu is mainly associated with the proteins and low molecular weight materials as was noted earlier. The cell walls also provide an important sink.

Table 88 illustrates the Cu distribution in the control plants. Very little Cu was found in the Parys (Cu tolerant) plants. Higher concentrations were found in the other populations i.e. 87 ug/g for Merlin and 100 ug/g for Goginan. The shoot concentrations were lower than the root concentrations.

The Parys root Cu was located entirely in the soluble protein fraction. Much of the Merlin root Cu was in the protein/amino acid fraction (~78%), the remainder being found in the soluble protein (~10%), low molecular weight (~1%) and insoluble pectate (~10%). The Goginan Cu was associated with the cell wall. The shoot Cu for all the populations was found mainly in the soluble protein fractions i.e. Parys ~ 46%, Merlin 100% and Goginan ~ 56%. The remaining Goginan Cu was also associated with protein i.e. the protein/amino acid fraction. The remaining Parys Cu was located in the cell walls.

Under these conditions, i.e. lower Cu uptake, the Cu is predominantly associated with the protein (Table 123).

Table 123 : Summary of the fractionation of Goginan, Merlin and Parys grown in non-amended culture solution. Cu content

	Percentage of Total Cu					
	Goginan		Merlin		Parys	
Fraction	Root	Shoot	Root	Shoot	Root	Shoot
Proteins/amino acids	-	100.0	88.5	100.0	100.0	45.5
Polar/low M(r) materials	-	-	1.3	-	-	-
Cell wall components	100.0	-	10.3	-	-	54.5

Table 89 shows the Cu distribution in Goginan, Merlin and Parys grown in Cu amended culture solution.

Copper was readily taken up by all three populations. The Cu-tolerant Parys plants in particular contained high Cu levels, as shown in Table 124.

Table 124 : Total Cu content of Goginan, Merlin and Parys plants grown in nutrient solution amended with different metals

	Cu concentration µg/g			
TABLE NO.	GOGINAN	MERLIN	PARYS	TREATMENT
88	100	87	18	CONTROL
87	273	185	147	Zn
84	58	277	594	Pb
89	1270	1190	1885	Cu

The shoots contained lower Cu levels than the roots. The proportion of Cu transported to the shoots by the Parys plants was lower than that of the other two populations - i.e. 22% of the total Parys Cu was present in the shoots compared with ~ 27% of the Merlin and ~ 30% of the Goginan Cu.

The Cu-tolerant Parys plants were found to contain much of the root Cu in the protein/amino acids (~ 84%) fraction, the remainder of the Cu was also associated with a variety of fractions : pigments (~4%), soluble protein (~ 2%), low molecular weight materials (~10%) and cell walls (~ 0.4%). The Merlin root Cu was located in six fractions, namely the protein/amino acid (~ 35%), polar low molecular weight materials (~ 24%), low molecular weight materials (~ 23%), soluble protein (~1%), pigments (~ 6%) and cell wall (~ 10%) fractions. A greater proportion of the Goginan root Cu was associated with all seven fractions; cell walls (~ 52%), and a lower proportion with the proteins - protein/amino acids ~ 15%,

soluble protein ~ 0.2% - Cu was found in the pigment (~3%), low molecular weight materials (~2%), polar low molecular weight materials (~21%) and polysaccharide (~6%) fractions.

The Parys shoot Cu was also associated largely with the protein/amino acid fraction (~50%). The cell walls contained some (~15%) and the rest was found in the polysaccharides (~16%), soluble protein (~0.2%), low molecular weight materials (~10%) and the pigments (~8%). The Merlin shoot Cu was located predominantly in the cell walls (~44%). The protein/amino acid fraction contained ~24%, and the polar (~9%) and low molecular weight materials (~13%), soluble protein (~2%), polysaccharides (~1%), and pigments (~7%) contained the remainder. For Goginan the cell walls held ~45%, and the protein/amino acids fraction ~22%. Low molecular weight materials were found to contain ~7%, polysaccharides ~10%, soluble protein ~4% and pigments ~13%.

The proteins are again major sites for Cu accumulation - especially in the Cu-tolerant Parys plants. The cell wall fractions were sites of major metal accumulation for Goginan and Merlin, while the low molecular weight materials were of importance (see Table 125).

Table 125 : Summary of the fractionation of Goginan, Merlin and Parys grown in Cu amended culture solution. Cu content

	Percentage of Total Cu					
	Goginan		Merlin		Parys	
Fraction	Root	Shoot	Root	Shoot	Root	Shoot
Pigments	3.1	13.3	6.2	6.5	4.3	8.3
Proteins/amino acids	15.4	26.0	36.8	26.1	85.6	50.4
Polar/low M(r) materials	22.8	7.0	46.9	22.3	9.7	10.1
Cell wall components	52.2	45.0	10.1	43.9	0.4	14.9
Polysaccharides	6.4	10.0	-	1.2	-	16.3

Table 90 shows the Zn distribution in Goginan, Merlin and Parys plants grown in Cu amended culture solution.

The Zn concentrations were high in the three populations. The Parys population exhibited the greatest Zn content of the three species. Both Parys and Merlin were found to have increased Zn uptake in the presence of Cu, but Goginan showed a decreased Zn content when compared with the controls. The shoots of all three populations contained less Cu than the roots.

Most of the Parys root Zn was located in the polar low molecular weight fraction (~ 44%). The soluble protein contained ~ 19% and the cell wall~ 26%. The remainder was located in the pigment (~ 5%) and polysaccharide (~6%) fractions. The polar low molecular weight fraction was a site of Zn accumulation (~40%) for Merlin roots, and the protein fractions - soluble protein (~ 21%) .and protein/amino acids (~ 21%) - were also important. The cell wall constituents contained the remainder. For Goginan, the cell wall components were the main sites of metal accumulation, ~ 79%. The polar low molecular weight materials contained ~ 7% and the soluble protein ~11%. The remaining 3% was located in the polysaccharides.

The Parys shoot Zn was found in the polar low molecular weight (~ 22%), soluble protein (~ 26%), cell wall (~ 43%) and pigment (~10%) fractions. In Merlin shoots there was a greater proportion of Zn associated with the soluble protein fraction (~ 43%). The cell wall contained ~ 56% and polar low molecular weight materials only 0.4% while the pigments also contained ~ 0.4%. For Goginan, the cell walls contained ~ 54%, the protein/amino acids ~ 24%, soluble protein ~ 1% and polar low molecular weight materials ~ 18%. The remaining 3% was located in the pigment fraction (Table 126).

The three fractions previously found to be the main sites of Zn accumulation were again found to contain most of the Zn i.e. the cell

Table 126 : Summary of the fractionation of Goginan, Merlin and Parys grown in Cu amended culture solution. Zn content

	Percentage of Total Zn					
	Goginan		Merlin		Parys	
Fraction	Root	Shoot	Root	Shoot	Root	Shoot
Pigments	-	2.8	-	0.4	5.2	9.6
Proteins/amino acids	10.9	25.1	42.0	43.2	-	25.8
Polar/low M(r) materials	7.3	17.9	39.9	0.4	62.7	21.5
Cell wall components	79.2	54.1	18.1	55.9	26.1	43.3
Polysaccharides	2.5	-	-	-	5.8	-

walls, polar low molecular weight materials and proteins. The polar low molecular weight materials were the most important for the plants with the highest Zn content i.e. Parys. The Goginan plants were found to accumulate the least Zn - the cell walls being the major sites, and Merlin being intermediate in Zn content - and contained Zn in all 3 of the major fractions.

The plant extraction experiments have shown that all four metals were readily taken up from the nutrient solution, when present at high concentrations, i.e. restricted uptake did not occur. Under these conditions restricted transport to the shoots occurred in all three populations. For Cu however, at lower concentrations, mobility within the plant was greater. The distribution of Cu between roots and shoots has been found to vary widely with plant species, and cultivars and with environmental conditions. As the solution Cu concentration increases, the root-Cu concentration of most species increases much more rapidly than that of the aerial parts (64). Copper-tolerant clones of A. tenuis were not able to take up as much Cu^{2+} from extremely dilute solutions of

Cu^{2+} as were susceptible clones. This phenomenon was observed for the control plants - the Parys population was found to contain less Cu than the other two populations. These observations may be interpreted as indicating a diminished capacity for Cu uptake in the Cu-tolerant clones which illustrates a facet of an exclusion mechanism.(31). However, the Parys plants were found to accumulate a higher Cu concentration than the other two populations, when the nutrient solution was amended with Cu. Uptake by the Parys plants may therefore be stimulated by high Cu solution concentrations.

Zn uptake showed a different pattern, being accumulated to high concentrations even when present at low solution concentrations. Other workers studying A. tenuis noted that the Zn concentrations in the plants were in excess of the concentration in the solutions after only seven days (27), which may indicate that the evolution of metal tolerance has incidentally increased the requirements of tolerant plants to the metal to which they are adapted, i.e. a 'need' for the metal (69). The Cu-tolerant Parys also showed high Zn accumulation. In some species it has been found that selection for Cu tolerance alone gives rise to increased tolerance to other metals also (64) - the Parys plants were apparently able to tolerate all four of the metals studied. If a similar phenomenon to that of metallothionein in mammals does occur in plants, the production of a metal-binding protein would help to explain the ability of some plants to tolerate more than one metal (31).

From the extraction results obtained from Goginan, Merlin and Parys, it appears that at high metal concentrations, in general :

1. Cd is predominantly associated with protein
2. Pb is predominantly accumulated at the cell walls
3. For Zn accumulation the polar low molecular weight materials are important

4. Cu shows a great association with proteins, and low molecular weight materials (possibly amino acids).

The cell walls were found to be sites of accumulation for all four metals. The cell wall accumulation could occur :

1. before activation of the proposed main tolerance mechanism e.g. prior to production of sufficient organic acids
2. after saturation of the main tolerance mechanism
3. as part of the tolerance mechanism.

For Pb the latter appears to be the situation. Other studies have noted Pb deposition in the cell walls of hydroponically grown corn, possibly as a Pb-phosphate complex (366).

Metal interactions were found to occur. At high Pb and Cu solution concentrations, an increase in Zn content was noted for the Merlin and Parys plants and a decrease in the Zn content for Goginan - the Pb effect was slight. High solution concentrations of Zn and Pb resulted in increased Cu contents of Merlin and Parys plants - and for Goginan at high Zn levels. A decrease in Goginan Cu content was observed at high Pb levels. Such interactions raise questions concerning the nature of the tolerance mechanism - is it the ability of the plant to overcome decreased essential metal levels that permits survival or is it the ability to counteract toxic concentrations of one metal by increasing the uptake of another? In any case, the metals have an effect on each other and the plants studied here apparently use mechanisms involving Pb, Zn and Cu - as reported by Coughtrey and Martin for H. lanatus (139).

g) Characterisation of plant metallothionein-like proteins

Because of the high proportion of metal associated with the protein fraction, determined from the plant fractionation studies, and recent reports on the isolation of plant metallothionein-like proteins (154 to 160), a more detailed investigation of the protein-metal association was attempted.

Table 91 shows that Zn, Cd, Pb and Cu were not detectable (Cytochrome C contained 0.7 µg/ml Zn) in the reagents or materials used.

Agrostis tenuis

The UV absorption spectrum of the material extracted from Parys (Cu-tolerant) roots showed absorption at 280 and 250 nm (Figure 74 and Appendix 2). The absorption at 280 nm was approximately half that at 250 nm. High absorptions were also found at 230 and 240 nm (Appendix 2). The UV absorption zone and Cu content were found in the same fractions (Figure 74). By means of gel permeation chromatography, the molecular weight was estimated to be 4,600 daltons. The material was partly eluted from the DEAE-cellulose column before application of the NaCl gradient. The remaining material was eluted at increased NaCl concentrations (Figure 75).

For Goginan (Pb/Zn tolerant) the absorption at both 250 and 280 nm was low. With fractions 18 and 19 the molecular absorption at 250 nm increased slightly - higher absorbances were also recorded at 240 and 230 nm. The Cu content of the extracted material was eluted at a number of fractions - the highest Cu concentration being found at fractions 13 to 18. The molecular weight of the extract (eluted at fractions 12 to 14) was greater than that of bacitracin (eluted at fractions 8/9 on Sephadex G25). The Cu containing material was eluted from the DEAE-cellulose column before application of the NaCl gradient.

Little is known concerning the structure and function of sulphur-rich metalloproteins which do not bind iron under physiological conditions. A characteristic feature of these proteins is their ability to bind Zn, Cd, Hg and Cu. At present, it is widely accepted that the unique properties are due to a coordination of the metal ions to the cysteine-sulphur atoms. A great variety of Zn-S and Cu-S proteins are expected to exist. Metallothioneins, from all sources, thus far examined, are deficient in aromatic amino acids. The typical absorption bands in the 260-290 nm region due to aromatic side chains, therefore, are absent. in the metal-free protein thionein. Binding of metal ions to thionein leads to UV spectra which are characteristic for the metal-sulphur bond (251). The absorption spectrum of (Cd, Zn)-thionein has a broad shoulder at 250 nm. Zn-thionein, however, has an absorption maximum around 210 nm with little or no absorption at 250 nm. Thus, the molar extinction coefficient of a (Cd, Zn)- thionein may be expected to vary with the Cd:Zn ratio of the material. The absorbance of Cu-mercaptides is at 330 nm (150), but Cu-thionein from yeast exhibited a shoulder at 270 nm tailing off into the visible region (151). (The molar extinction coefficient would also be expected to vary with varying metal ratios.)

The material extracted from the Parys roots exhibited the characteristic low absorption at 280 nm. The higher absorption at 250 nm corresponds to that obtained by Rauser and Curvetto (154) for a Cu-thionein extracted from Cu-tolerant Agrostis gigantea. The protein extracted by these authors was resolved into two fractions, one of which (fraction B) was found to have the following UV absorption spectrum (Figure 110).

The material isolated in the present work from A. tenuis (Parys) also exhibited a high absorption at 230 to 250 nm. Rauser and Curvetto's fraction B protein contained a small percentage of bound Zn. Since

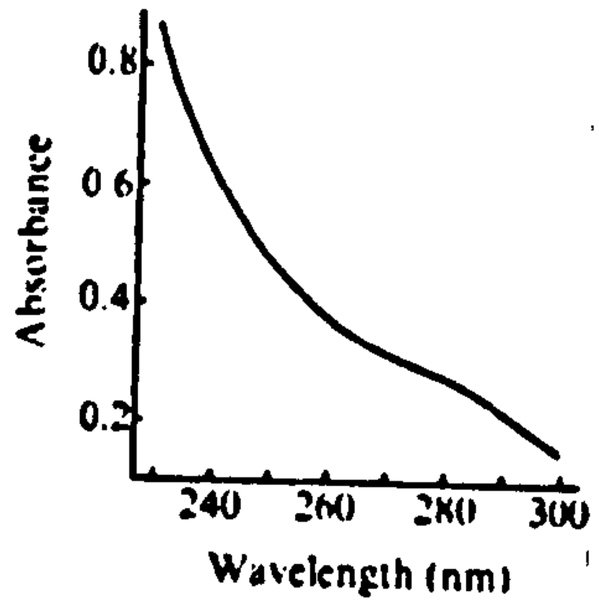


Figure 110 : UV absorption spectrum of Fraction B protein isolated from A. gigantea (154)

Parys plants were found to accumulate high Zn levels, when grown in Cu amended culture, the presence of Zn in the isolated material is possible. Studies with certain mammalian metallothioneins have suggested that Zn^{2+} is essential for the accumulation of thionein-bound Cu (150). If this is also true for the higher plants, it may explain the high Zn concentrations found in the Parys plants.

Mammal metallothioneins have been consistently reported as possessing molecular weights of between 10,000 to 12,000 daltons when determined by gel filtration. Values of 6,600 daltons (derived from amino acid composition) have been reported by other authors. It seems, therefore, that the metallothioneins in general are polydisperse and consist of several forms of approximately the same molecular weight (150). It is proposed that the proteins consist of a single polypeptide chain (~ 6000 daltons) (351). The Parys material was found to have a much lower molecular weight of approximately 4600 daltons which is similar to that determined by Linss et alia (159) for yeast Cu-thionein (molecular weight 4800). The Fraction B protein isolated by Rauser and Curvetto (154) was found to have lesser bands (major band was at 14,400), one of which occurred at 5400 daltons (by gel electrophoresis). The Cu-chelatin has

been isolated from various eukaryotic sources and has a low molecular weight of ~ 8000 assuming a globular conformation (160,367). Rat Cu-chelatin is strongly adsorbed by DEAE-cellulose, and is not eluted even at high ionic strengths. Under aerobic conditions (Cu,Zn)-thioneins in which the Cu:Zn ratio is high, behave in the same way (150). The Fraction A protein isolated by Rauser and Curvetto was not readily adsorbed by DEAE, but the fraction B protein was eluted only after addition of NaCl. Similar fractionations have been observed for animal (Cd,Zn)-thioneins. Resolution of the metallothionein from the kidney of Cu poisoned sheep isolated by ion-exchange chromatography yields 3 sub-fractions, one of which does not bind to the column. As with (Cd,Zn)-thionein from rat liver, the relative proportions of these subfractions are not constant, but vary between preparations from different animals. (150). The Parys material was found to consist of more than one fraction. Most of the Cu was contained in material that was not adsorbed by DEAE cellulose, while a much smaller proportion was found to be eluted at high NaCl concentrations. The material isolated from the Cu treated Parys roots, thus shows the characteristics associated with metallothioneins. Further investigation is required to identify the material.

The Goginan extract did not, on the whole, exhibit the characteristic low 280 and high 250 nm absorbances, and the Cu content was eluted from the preparative gel filtration column at several fractions. The molecular weight of the extract was lower than that obtained for Parys and all the Cu containing material was eluted from the DEAE column, before application of the NaCl gradient. For two of the fractions, some binding of the Cu occurred, since a higher absorption was found at 230 to 250 nm which may be due to the presence of a small quantity of metallothionein, or other Cu-protein or amino acid associations. Although treated with a lower Cu concentration than the Parys plants, stunting of root growth occurred,

suggesting that the differences in tolerance are related to the ability of the tolerant plants to synthesise the metal binding protein.

Silene vulgaris

The UV absorption spectrum showed a low absorption, at both 280 and 250 nm, but a high absorption at 220 to 230 nm (Figure 78 and Appendix 2). The metal zones and high absorption zones were similar, although not corresponding as closely as the Cu and high absorption of the Parys gel filtration fractions. Pb was eluted at two points - one was close to the void volume, the other was eluted with the Zn at fractions 13 to 20. The molecular weight of the extract was found to be similar to that of bacitracin (both eluting at fractions 8 and 9) i.e. approximately 1450 daltons (Appendix 2). Much of the Zn containing material was not adsorbed by the DEAE column, which was also true for some of the Pb containing material. The remaining Pb was eluted after application of the NaCl gradient. The maximum absorbance occurring between fractions 7 and 13 did not correspond to the metal elution zones (Figure 79).

High absorbance at 210 nm and low absorbance at 250 nm is characteristic of Zn-thionein. The presence of Cd may explain the high 220 nm absorbance. The low molecular weight indicates that the material is a polypeptide rather than a protein. As Zn^{2+} is less firmly bound by thionein than is Cd, Hg or Cu, native Zn-thionein even if fully loaded in vivo, may be expected to lose some Zn^{2+} during gel permeation and other purification techniques (150). Partially oxidised forms of metallothionein may therefore result, which would be resolved into two or more components by ion exchange chromatography. Other investigations have revealed minor differences in the amino acid compositions of the multiple forms of a particular metallothionein (150).

The binding ability of Pb^{2+} to SH groups is somewhat weaker than that

of Zn, Cu, Cd or Hg (7) (see Table 127).

Table 127 : Stability constant values for some metals with cysteine at 25°C

Metal ion	Equilibrium		Log K	Ionic strength	Reference
Hg^{2+}	ML/M.L.		45.4	-	45
Cu^{+}	ML/M.L.		19.2	0.01	368
Cu^{2+}	a				369
Zn^{2+}	$ML_2/M.L.^2$	~	18.12	0.1	370
Zn^{2+}	$ML_2/M.L.^2$		19.39	3.0	370
Cd^{2+}	ML/M.L.		12.88	3.0	370
Cd^{2+}	$ML_2/M.L.^2$		19.60	3.0	370
Pb^{2+}	ML/M.L.		12.21	3.0	370
Pb^{2+}	$ML_2/M.L.^2$		18.57	3.0	370

a = reduction of metal ion by ligand

The effects of the isolation procedure may explain the slight differences between the metal and absorption zones (Figure 78) although for Thlaspi alpestre the Zn, Cd and high absorptive zones (of preparative gel filtration) coincide (Figure 80).

Very little (~ 0.1 µg/ml) of the Zn contained in the high speed centrifuge supernatant (total Zn ~ 1.8 µg/ml) was found to be associated with the high absorption zones. Similarly for Pb 1.6 µg/ml was found in association with the high absorption zones compared with 7.1 µg/ml in the high speed extract. The remaining Zn and Pb is diluted in the gel filtration procedure (and was not detected by FAAS) or is collected from

the column at a much later stage i.e. is present as very low molecular weight materials. Determination of low Zn or Pb concentrations by DPASV was precluded because of the organic nature of the sample. GFAAS was not used at this time due to the likelihood of fairly large Cl^- interference. An association with the low molecular weight fraction was noted for Zn and Pb in the fractionation studies carried out on Merlin, Parys and Goginan plants. The percentage of the metal found in the high UV absorption material and the concentration found in the high speed extract is given in Table 128.

Table 128 : Percentage of metal recovered in the high UV absorption material

Sample	Metal	High speed extract	"Recovery" %
Goginan	Cu	1.5	80
Parys	Cu	7.5	72
Silene	Zn	1.8	6
	Pb	7.1	23
Thlaspi	Zn	128.0	9
	Cd	0.05	100

From Table 128, Zn and Pb are not associated with the high absorption material to any great extent, unlike Cu which is mainly recovered and Cd which is entirely recovered with the material. The protein/amino acid fractions were found to be more important for Cu and Cd (the latter in particular) in the fractionation studies previously carried out (page 242). The Zn and Pb found to be recovered with the absorbing material may be due to interaction with SH containing materials

other than metallothionein, but which share the metallothionein characteristics of low absorption at 280 nm (no aromatic side chains) and high absorption at 220 nm (Zn, Cd thionein). The low molecular weight, however, points to a polypeptide rather than a protein.

Thlaspi alpestre

Figure 80 : both the Zn and Cd recovered coincide with the high 220 nm absorbance zone. The material had a higher molecular weight (i.e. > 1450 daltons) than that of Silene but was similar to that of the Goginan material (Appendix 2). The Zn containing material was eluted as two fractions after passage through the DEAE cellulose column - one was not absorbed by the column, the other only being eluted after addition of the NaCl gradient. The Zn and absorbance zones overlapped, whereas the small Cd zone coincided with the 200 nm absorbance zone, and was eluted at the commencement of the NaCl gradient (Figure 81).

Again, the material exhibits the absorbance characteristics of metallothionein, but has the low molecular weight of a polypeptide. Further investigations - such as amino acid analysis - are required to resolve the nature of this metal binding material.

h) Statistics

Standard Addition Method : HMDE and RDE

An example of a standard addition scan for DPASV-HMDE is shown in Figure 82. The influence of blank contamination can clearly be seen - the Cu blank value in particular is high, and demonstrates the importance of using the limit of detection (expressed as the concentration or quantity derived from the smallest measure that can be detected with reasonable certainty for a given procedure) rather than the sensitivity (the response

function expected in the absence of interference is $\hat{y} = f_o(c)$, and the sensitivity is defined as the partial derivative of $\frac{\partial y}{\partial c}$ (371)) which refers only to the performance of the apparatus (372).

Table 93 shows the results obtained for six replicate standard additions, together with the mean, standard deviation and standard error. From the values obtained the Cu determination was found to be the most precise, having the lowest dispersion (Precision = degree to which data generated from replicate or repetitive measurements differ from one another. Statistically this concept is referred to as dispersion (373)). The Cd determination was found to be the least precise, having a standard error of 1.65 i.e. precision decreased in the order : Cu > Zn > Pb > Cd.

Tables 94 to 97 depict the effect of the deposition time on peak height, and is expressed graphically in Figures 83 to 86. The longer the deposition time, the higher is the metal concentration in the mercury drop. Thus, long deposition times lead to increased sensitivity. However, the longer the period of electrolysis the greater quantity of metal diffuses into the drop. As the metal requires a finite time to diffuse back to the mercury/solution interface, broadening of the stripping peaks results, and possible contamination of the mercury in the capillary can occur. Added to this, the risk of drop fall increases with longer deposition time.

The reproducibility (defined as that difference between single and independent test results as would be exceeded in the long run in only one case in twenty in the normal and correct operation of the test method) was found to decrease as the deposition time increased for Zn and Cu, i.e. standard error increased from 0.01 to 0.03 and from 0.01 to 0.06 respectively. Thus the reproducibility of the Cu determinations in particular decreased with increased electrolysis time. For Cd and Pb the standard errors remained low (at approximately 0.01) at all deposition times.

From the regression line equations, the gradient values (in cm/min) increase in the order : Pb < Zn < Cd < Cu, i.e. Cu shows the greatest peak height increase, with increasing deposition time. The increase in sensitivity was least for Pb.

Figure 87 presents the differential pulse stripping curve for Cd, Pb and Cu at an RDE. Zn was not determined by this method for two reasons :-

1. the Zn concentrations of the samples were usually high, not necessitating the use of a sensitive method
2. the pH found to produce the best results for Cd, Pb and Cu, 2.5, gave rise to interference between the Zn and hydrogen reduction peaks.

Table 98 depicts the mean, standard deviation and standard error found from six replicate standard additions. The Cu determination was the most precise and Pb the least - differing slightly from the HMDE method in that Cd was found to contain a greater error than Pb. The HMDE standard error values were greater than for the RDE.

Table 89 demonstrates the effect of deposition time on the peak height, which is also expressed graphically in Figures 88 to 90. The TFME has the advantage of no drop displacement, but the greater the deposition time, the greater the probability of changes in the electrode surface. The reproducibility of the HMDE was found to be higher. However, the RDE gradient values are greater than those obtained for the HMDE regression lines, indicating the greater sensitivity of the RDE technique.

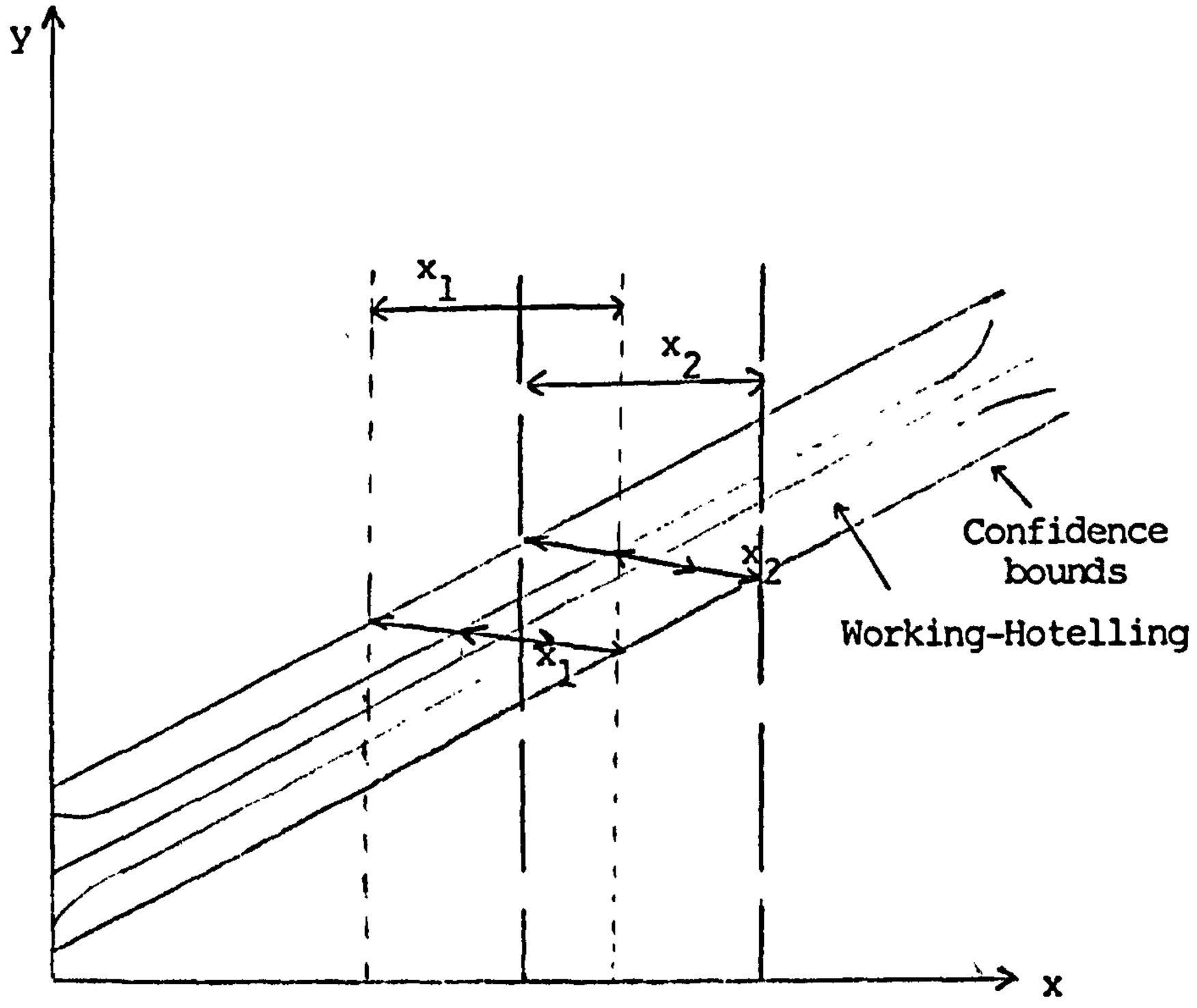
Confidence Bounds and Working-Hotelling

Tables 101 to 108 and Figures 91 to 101 display the regression line equations, and where possible the confidence bounds and Working-Hotelling 95% confidence region, for the FAAS, HMDE, RDE and GFAAS calibration lines.

The limits containing μ with a probability of 0.95 are 95%

confidence limits i.e. there is a 1 in 20 chance of a value lying outside the limits.

The Working-Hotelling bounds include the true value of the abscissa variable 90% of the time, a statement that can be made with 95% confidence (290).



The narrower band of the Working-Hotelling interval permits easier identification of differences between two points (x_1 and x_2, say), whereas the broader confidence intervals for x_1 and x_2 overlap.

In general, atomic absorption spectrometric calibrations are initially a straight line becoming progressively more curved at higher concentrations. Effects such as ionisation and flame reduction kinetics may cause the curve to have a more complex shape with points of inflection and even regions of negative slope (374). The analytical measurement precision also diminishes with decreasing concentration and tends to become poorest as the detection limit of the measurement method is

Table 129 : Sensitivity, Detection Limit and Relative Precision of FAAS, HMDE, RDE and GFAAS

Figures	Method	Sensitivity (Digital reading or peak height/unit concn.)		Detection limit	Decreasing order of precision
91 to 94	FAAS	Zn	b = 8.0	0.4 µg/ml	
		Cd	b = 9.1	0.1 µg/ml	Cu > Pb
		Pb	b = 2.1	0.1 µg/ml	> Zn > Cd
		Cu	b = 2.4	0.1 µg/ml	
95 to 98	HMDE	Zn	b = 1.8	1.0 ng/ml	
		Cd	b = 2.6	1.0 ng/ml	Pb > Cu =
		Pb	b = 1.1	1.0 ng/ml	Zn > Cd
		Cu	b = 1.6	2.0 ng/ml	
99 to 100	RDE	Cd	b = 0.3	0.8 ng/ml	
		Pb	b = 0.5	0.4 ng/ml	Cu > Pb > Cd
		Cu	b = 0.3	0.7 ng/ml	
101 to 102	GFAAS	Cd	b = 0.1	0.8 ng/ml	
		Pb	b = 0.1	0.5 ng/ml	Cu = Cd > Pb
		Cu	b = 0.1	0.5 ng/ml	

approached (375). Figures 91 to 102 demonstrate the curvature of the confidence bounds and Working-Hotelling confidence regions.

The sensitivity of the FAAS method for the four metals was found to decrease in the order of Cd > Zn >> Cu ~ Pb. The influence of contamination on the detection limit was also observed. Sources of blank contributions include leaching from containers, and ancillary materials such as filters, atmospheric deposition and the analyst. Lead is perhaps the most ubiquitous of all environmental contaminants (375) and this fact was demonstrated by the detection limits obtained (ranging from 0.1 μg Pb/ml for FAAS to 0.4 ng Pb/ml for RDE) resulting from high blank values. Lead's widespread distribution is based on its extensive use in fuels. Data have shown that a clean room or clean hood will reduce the air lead concentration by about a factor of a thousand to 0.0001 - 0.0005 $\mu g/m^3$ (375). The presence of Zn in plant material means that Zn will have a widespread distribution - especially in paper products (e.g. tissue paper 48.8 μg/g (255)). Copper also present in plant tissue can occur as a major contaminant as a result of copper piping. The distribution of Cd would be expected to be more localised than that of the other three elements, because of its association with Zn and its ready uptake by plants ensures its presence in the laboratory. Sample solutions become contaminated due to leaching of Cd from some types of micropipette plastic tips (176). Cd was found to have detection limits ranging from 0.1 μg/ml for FAAS to 0.8 ng/ml for RDE. The low precision found for Cd determinations may be due to the strong dependence of the absorption of Cd upon lamp operating current (168).

The sensitivity of the HMDE method for the metals decreased in the order Cd > Zn > Cu > Pb i.e. similar to that of FAAS. The lower precision obtained for Cu, Zn and Cd compared to Pb may be due to the formation of intermetallic compounds as is well known for Cu/Zn (259,204).

The sensitivity of the RDE technique was similar for Cd, Pb and Cu - the sensitivity for Pb being slightly better than for Cd or Cu. The mercury film tends to become unstable (leading to changes in sensitivity) and must be frequently renewed - a time consuming process. A further cause of loss of sensitivity is contamination of the electrode surface by adsorption, which occurs if measurements are made in samples containing colloidal or suspended matter (376). For routine analysis the HMDE was found to be the easier method to use. The RDE surface, not only required frequent re-polishing, but was more sensitive to the presence of organic substances than the HMDE, leading to distorted scans and unquantifiable results. The popularity of the HMDE is understandable - in view of its robustness; the reproducibility being better than that for the RDE. The formation of intermetallic compounds is again a problem at the TFME. For concentrations greater than 100 ng/ml Cd and 200 ng/ml for Pb there is a decrease in sensitivity (259).

The sensitivities for Cd, Pb and Cu as determined by GFAAS were similar. The low precision found for the Pb determinations may be due to interferences arising from alkali-metal or alkaline earth chloride contaminants (176).

i) Sources of Contamination

Table 109 shows the levels of Zn, Cd, Pb and Cu in materials that are often used during an analytical procedure. The results illustrate the problem of contamination. The filters were all found to contain varying levels of Zn, Cd, Pb and Cu - Zn in particular was present at high levels (up to 14.8 μg/g in 0.1 μm pore size filters). Cu was also found at high levels (up to 27.8 μg/g) but was not as widely distributed as Zn. The Zn and Cu concentrations of the handled Whatman 541 filter

paper were increased when compared to the non-handled papers (e.g. an increase of 1.0 µg Zn/g and of 0.2 ng Cu/g). The Cd and Pb levels were undetectable by GFAAS. However, all analysts ingest nominally 50-100 µg Pb daily and a portion of this is excreted in sweat. More importantly, particles accumulate on skin and clothing from the air, thus emphasising that surfaces likely to come into contact with a sample or solution should not be handled (375). Furthermore, the results illustrate the necessity of precleaning filters. The filter separators are another source of filter contamination, levels of 20.8 µg Zn/g, 5.3 ng Cd/g, 4.8 ng Pb/g and 27.8 ng Cu/g being found for these materials.

Tissues are often used to wipe pipettes or to rest glassware on. Both the blue towel paper and Mediwipe tissues were contaminated and should be avoided, wherever possible. The blue towel paper, especially, is a potential source of major contamination - containing up to 56.2 µg Zn/g, 3.0 ng Cd/g, 219.3 ng Pb/g and 5.0 ng Cu/g.

PART IV

CONCLUSIONS

CONCLUSIONS

Of the purification methods studied isothermal distillation proved to be a simple, economical means of producing high purity acids. The method is dependent on the purity of the water employed and therefore must be used in conjunction with Amberlite IRC 718 resin or thermal distillation. Mercury cathode electrolysis was suitable for the purification of large volumes of background electrolytes.

Samples collected from sites long since abandoned for mining were found to be still heavily contaminated with metals. In general, the metal level was found to decrease with a decrease in size fraction, and to increase with decreasing pH. Zinc was found to be present as both labile and "bound" metal, in samples collected from the Rheidol and Ystwyth rivers - the majority being associated with dissolved organic matter, colloidal and mineral material. The contamination increased as water flowing over the dumps leached metal from the waste material. A carbonate trap proved to be an efficient means of controlling the pollution.

The lead in the Mineries Pool sediment was mainly associated with particles whose dimensions were between 0.1 and 5 μm - the metal passing through the 0.45 μm filter was principally ASV-labile at pH 3 i.e. metal-organic complexes. Approximately half of the Cu present in the Caradon sediment was particulate in form (i.e. > 0.45 μm). Again the metal was present as both labile and complexed forms. The Garston dock sediment consisted of a range of particle sizes of which the < 20 μm fraction formed only a small proportion of the total. The Zn and Cd, and Pb and Cu showed similar trends in their associations with the different size fractions, possibly due to the presence of different minerals or organic complexation.

Rock from the Ystwyth site was found to be easily leached by a pH 7 extractant and that additional further leaching would occur at lower pHs.

The slag samples collected from Lawrence Weston were identified as belonging to Roman smelting operations. The low metal levels determined were indicative of re-smelting at a later date.

Experimental results obtained from the extraction of model samples demonstrated that samples with high organic content must be treated with care. The H_2O_2 used should be added as 1 ml aliquots to prevent sample loss due to the bubbling effect produced. Furthermore, it is advisable to carry out the organic extraction before that of the reducible extraction since the extractants used for these two phases tend to attack both fractions. The summed totals of the extracts and the HF/HNO_3 totals were in general found to agree well.

Extraction of the '4 metal' peat by the Tessier scheme indicated the following order of organic complexation :

Pb > Cu > Zn > Cd

and the relative order of availability was :

Cd > Pb > Zn > Cu.

The Pb and Cu were the most readily bound by organic material, while the ready availability of Cd has worrying implications for plant uptake.

The scavenging efficiency of ferric hydroxide was demonstrated by the results obtained from the fractionation of lead contaminated ferric hydroxide. For this type of sample, the reducible fraction must be extracted before the organic extraction.

The NBS SRM 718 Orchard leaves extracted by the Tessier scheme gave some idea of the accuracy of the scheme. An indication of the metal binding ligands present was also obtained. The mobility of the metals decreased in the order :

Cd > Zn > Cu ~ Pb.

The results point to a possible Zn-organic acid association, whereas the Pb was located in the cell walls and the Cu was mainly associated with protein.

The Hallen litter was found to contain much higher levels of Cd, Cu, Pb and Zn than the litter collected from Wetmoor. The residual fraction was the most important for the Zn found in the Hallen sample, while the Cd was present in the readily available portion. The order of metal availability at the two sites was :

Cd > Zn ~ Pb > Cu Hallen

Pb > Zn > Cd > Cu Wetmoor

The Cu was least available due to its ability to form strong associations with clay, organic matter and hydrous oxides. The absolute concentrations of metal in the Hallen organic fraction followed the order :

Pb > Cu > Zn > Cd

and for Wetmoor :

Cd > Cu > Pb > Zn.

The differences between the two sites are thought to relate to a greater degree of decomposition occurring at the Wetmoor site.

Soils from Luckett and Callington were found to be contaminated with Cu, Pb and Zn and to a lesser extent Cd. The Cu was present in the less available forms (i.e. the organic and residual phases were important). Lead was contained mainly in the residual fraction. Zinc in the Callington soil exhibited a diverse distribution, being found in both readily available and less available forms. Only a small proportion of the Zn was readily available in the Luckett samples. Cadmium, present in the Callington sample C2 was readily available. The order of metal bioavailability, for the two sites, was :

Zn > Cu > Pb Callington

Cu > Zn > Pb Luckett.

The Hallen soil was contaminated with high levels of Zn, Pb and Cd. The Zn was mainly associated with the organic and reducible phases, Pb and Cu with the organic, reducible and residual fractions, while Cd was more readily available, being located in the organic and exchangeable fractions.

High Zn, Cd and Pb levels were also found in soils collected from Shipham, Bedminster and Charterhouse, reflecting the mining/industrial histories of these sites.

The "Quick" scheme provided a rapid means of determining the degree of metal availability, but it is necessary to carry out the organic extraction before that of the reducible, for samples of high organic content.

The UV spectra obtained for HA and FA were generally uncharacteristic. The intensity of absorption decreased as the wavelength increased.

The following diffusion coefficients and stability constants were determined for metal-HA/-FA complexes :

	DC (cm^2 s^{-1})
Cu/HA	1.40×10^{-6}
Cu/FA	1.89×10^{-6}
Pb/HA	2.60×10^{-6}
Pb/FA	2.50×10^{-6}
HA-Zn	1.44×10^{-7}

	K ML (mol^{-1} l)
Cu-HA	9×10^{5}
Cu-FA	9×10^{3}
Pb-HA	1.88×10^{5}
Pb-FA	6.22×10^{5}

The relative strengths of complexes of metals with both FA and HA follow the order :

$$Cu > Pb > Zn \sim Cd$$

The same sequence was found earlier for the association of the four metals with the organic fraction.

Cadmium was readily taken up into the roots of the Hallen and Midger Holcus lanatus, but was not readily transported to the shoots. From the results obtained by application of the Tessier scheme, the Cd was present as free ions, bound to proteins/organic acids and bound to the cell walls. It is possible that the ability of the Hallen H. lanatus to tolerate Cd is related to its greater Cd association with the ionic/protein/organic acid fraction.

The "Quick" scheme results for the Hallen shoots indicate much of the Pb and Cu present as cell wall bound metal. The Zn was mainly located in the exchangeable/carbonate fraction.

The Cu- and Cd-peat samples were successfully fractionated by the Farago scheme. The Cd was found in all fractions, but for the most part was firmly bound, being leached only by the more severe conditions of the later stages of the scheme, i.e. located in the cell walls as well as being found in the protein fraction. The Cu was also found in all fractions, and was combined with the cell wall constituents. However, a higher proportion of Cu was found in the low molecular weight and protein/amino acid fractions.

The Zn contained within the SRM 718 Orchard leaves was contained in the protein/amino acid and cell wall fractions. The Pb exhibited a similar pattern to that of Zn, but was associated with the cell wall components to a much greater extent. The majority of the Cu was found in the protein/amino acid fraction. The summed totals and acid digests agree - within experimental error - with the NBS values. The Zn

determined in the Hallen H. lanatus was also mainly present with the proteins/amino acids and cell wall components. Much of the root Cd was with the protein fraction S, and the Pb was again found with the proteins and cell walls. The Cd, Pb and Zn levels in the roots were higher than those of the shoots, indicative of restricted transport. The Cu was established with the proteins and cell walls with root and shoot levels being similar in value.

Investigation of Agrostis tenuis and A. canina samples collected from Callington and Luckett respectively showed that the metal distribution within these plants was similar i.e. Zn with low molecular weight materials, proteins and cell walls, Cd with proteins, Pb mostly in the cell walls and Cu in proteins and cell walls. The moss samples from these sites - Rhytidiadelphus squarrous, Callington and Ceratodon purpureus, Luckett - contained much of their metal content within the cell walls, in accordance with the uptake mechanisms exhibited by mosses.

Cadmium was readily taken up from amended nutrient solution by Goginan, Merlin and Parys plants. All three populations exhibited restricted transport of the Cd to the shoots - the Goginan plants in particular. The lowest Cd concentration was found in the Merlin plants - but this population contained the highest Cd shoot concentration. The Cd was located mainly in the protein/amino acid fraction of all the plants - and was even found in small quantities in this fraction of the cpntrol plants. Some Cd accumulation occurred in the cell walls. For Pb - which was also restricted to the roots - the metal was also mostly in the cell walls. Zinc was readily taken up, but remained at higher concentrations in the roots. The Zn was combined with proteins, polar/ low molecular weight materials and the cell walls. Copper (greatest concentration in the roots) was present in the proteins - this fraction was especially important in the Cu-tolerant Parys plants - polar/low

molecular weight compounds and the cell walls. Thus for these three populations all four metals were readily taken up from the nutrient solutions i.e. exclusion of the metal from the plant did not occur, but restricted transport did. The Cu-tolerant clones of A. tenuis were not able to take up as much Cu^{2+} from extremely dilute solutions of Cu^{2+} as were the susceptible clones. Copper uptake by the Parys plants may be stimulated by high Cu solution concentrations. The Zn uptake pattern was different, being accumulated at high concentrations even when present at low levels in the nutrient solution - possibly indicating a greater requirement for this metal. In general :-

Cd was predominantly associated with the proteins;

Pb was accumulated in the cell walls;

For Zn, the polar low molecular weight materials are important;

The Cu was associated with proteins and low molecular weight material;

The cell walls were important for all four metals.

Metal interactions were identified :-

A high level of Pb or Cu in the nutrient medium caused increased levels of Zn in Merlin and Parys plants, and decreased levels for the Goginan population (the Pb effect was slight).

A high level of Zn or Pb resulted in increased Cu in Merlin and Parys and also for Goginan plants in the case of Zn. The Cu level of the Goginan plants was decreased when the Pb concentration was high.

The plants studied apparently have tolerance mechanisms which involve Pb, Zn and Cu.

Material isolated from the Cu treated (A. tenuis) Parys plants showed characteristics associated with metallothionein. Such material was not recovered from the Pb/Zn tolerant Goginan (A. tenuis) plants exposed to a high Cu concentration. Investigations involving Silene

vulgaris and Thlaspi alpestre indicated the presence of a low molecular weight material, more like a polypeptide rather than a protein. Zinc and lead were not associated with the material to any great extent, unlike Cu which was mainly and Cd which was entirely recovered with the material.

The precision of the HMDE in DPASV decreased in the order :-

$$Cu > Zn > Pb > Cd$$

The reproducibility of the Zn and Cu measurements decreased as the deposition time increased. For Cd and Pb the standard errors remained low at all deposition times. From the regression line equations, the gradient values (in cm/min) increase in the order

$$Pb < Zn < Cd < Cu,$$

i.e. copper showed the greatest peak height increase with increasing deposition time. The increase in sensitivity was least for Pb.

The precision of the RDE in DPASV decreased in the order :-

$$Cu > Cd > Pb.$$

Although more sensitive than the HMDE, the RDE measurements were less reproducible. The HMDE was easier to use for routine analysis. The RDE required frequent polishing and was more sensitive to organic interference.

The sensitivity of FAAS followed the order :-

$$Cd > Zn \gg Cu \sim Pb,$$

whilst for GFAAS the sensitivity remained the same for Cd, Cu and Pb, and the precision was as follows :-

$$Cu \sim Cd > Pb.$$

Contamination from a variety of sources is possible - it is necessary to leach metals from membrane filters before use, and to avoid the use of tissue papers (heavily contaminated with metals) in trace metal analyses. Handling of materials should be kept to a minimum.

PART V

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APPENDIX I

$$\% \text{ Moisture content} = 100 \times \frac{\text{weight of sample taken} - \text{dry weight of sample taken}}{\text{weight of sample taken}}$$

Percentage moisture content of Goginan, Merlin and Parys control and Cd test

	%		%
Goginan shoots	85.3	Cd sample	84.8
Goginan roots	97.2	" "	97.6
Merlin shoots	82.8	" "	82.6
Merlin roots	96.4	" "	96.0
Parys shoots	86.4	" "	84.8
Parys roots	95.9	" "	96.0

Percentage moisture content of Goginan, Merlin and Parys : control, and Cu, Pb and Zn tests

	CONTROL	Cu	Pb	Zn
Goginan shoots	84.0	76.3	80.7	83.4
Goginan roots	79.1	89.7	94.4	95.6
Merlin shoots	83.4	75.3	78.7	77.9
Merlin roots	88.5	95.6	92.8	91.8
Parys shoots	89.2	76.9	81.2	81.6
Parys roots	92.0	86.7	96.0	93.6

APPENDIX 2

Data from Metal-Containing Plants

Parys Agrostis tenuis (Cu-tolerant)

a) High-speed supernatant

Wavelength (nm)	Absorbance	
210	1.41	
220	> 2	Cu content = 7.5 µg/ml
230	> 2	(corrected for volume
240	> 2	and wet weight = 6.6 µg/g)
250	> 2	Roots = 539.7 µg/g (DW)
280	> 2	Shoots = 214.9 µg/g (DW)

b) Fractions collected from Sephadex G-50 preparative gel filtration column

	Absorbance									
	Fraction number									
Wavelength (nm)	1	2	3	4	5	6	7	8	9	10
220	0.99	0.69	0.57	0.47	0.43	0.44	0.45	0.45	0.37	0.32
230	0.84	0.26	0.21	0.17	0.16	0.20	0.24	0.18	0.17	0.16
240	0.20	0.09	0.07	0.06	0.06	0.10	0.11	0.08	0.08	0.08
250	0.08	0.04	0.04	0.03	0.04	0.06	0.08	0.05	0.06	0.05
280	0.04	0.02	0.02	0.02	0.02	0.04	0.05	0.04	0.04	0.04

	11	12	13	14	15	16	17	18	19	20
220	0.40	0.44	0.94	2.15	> 2	2.11	2.14	2.16	2.11	2.15
230	0.18	0.19	0.38	1.52	> 2	> 2	> 2	> 2	> 2	> 2
240	0.09	0.10	0.18	0.44	1.21	> 2	> 2	> 2	> 2	> 2
250	0.06	0.08	0.13	0.23	0.48	0.42	0.55	0.65	0.59	0.46
280	0.05	0.06	0.09	0.17	0.34	0.20	0.26	0.29	0.29	0.32

Parys. G-50 fractions continued

	Absorbance									
	Fractions									
Wavelength (nm)	21	22	23	24	25	26	27	28	29	30
220	2.00	1.95	1.90	1.50	0.86	0.49	0.28	0.17	0.17	0.11
230	> 2	1.89	0.98	0.48	0.29	0.18	0.13	0.09	0.10	0.08
240	0.71	0.34	0.20	0.10	0.07	0.06	0.05	0.04	0.05	0.05
250	0.19	0.11	0.07	0.05	0.04	0.04	0.04	0.03	0.03	0.04
280	0.13	0.08	0.05	0.04	0.03	0.03	0.02	0.02	0.02	0.03
	31	32	33	34	35	36	37	38	39	40
220	0.10	0.09	0.13	0.18	0.08	0.08	0.09	0.08	0.05	0.11
230	0.08	0.08	0.08	0.10	0.07	0.07	0.07	0.07	0.06	0.08
240	0.05	0.06	0.05	0.05	0.04	0.04	0.07	0.07	0.06	0.05
250	0.04	0.04	0.04	0.04	0.04	0.03	0.03	0.03	0.03	0.04
280	0.03	0.03	0.03	0.03	0.02	0.02	0.07	0.07	0.06	0.03

c) Parys DEAE fractions

	Absorbance									
	Fractions									
Wavelength (nm)	1	2	3	4	5	6	7	8	9	10
220	1.99	1.05	0.54	0.77	2.10	1.99	1.99	1.99	1.91	1.88
230	> 2	0.37	0.22	0.39	1.44	> 2	> 2	> 2	> 2	> 2
240	0.18	0.19	0.14	0.24	0.65	1.02	1.43	1.37	1.28	0.90
250	0.27	0.15	0.12	0.19	0.44	0.31	0.32	0.29	0.20	0.14
280	0.16	0.10	0.08	0.14	0.31	0.19	0.18	0.16	0.09	0.07
	11	12	13	14	15	16	17	18	19	20
220	1.83	1.84	1.86	1.80	1.78	1.74	1.70	1.70	1.71	1.69
230	> 2	> 2	1.77	0.91	0.72	0.60	0.60	0.58	0.59	0.59
240	0.70	0.53	0.30	0.16	0.11	0.11	0.11	0.10	0.10	0.09
250	0.10	0.08	0.06	0.05	0.03	0.03	0.03	0.03	0.02	0.03
280	0.04	0.03	0.03	0.02	0.01	0.01	0.01	0.01	0.01	0.01

Parys DEAE fractions continued

Absorbance

Fractions

Wavelength (nm)	21	22	23	24	25	26	27	28	29	30
220	1.72	1.93	2.05	1.82	1.59	1.52	1.61	0.36	0.64	0.32
230	0.63	> 2	> 2	0.82	0.51	0.51	0.60	0.16	0.21	0.13
240	0.11	0.59	0.86	0.24	0.15	0.16	0.19	0.09	0.08	0.07
250	0.04	0.22	0.50	0.14	0.08	0.09	0.11	0.07	0.05	0.05
280	0.02	0.13	0.31	0.08	0.05	0.06	0.07	0.05	0.04	0.04

	31	32	33	34	35	36	37	38	39	40
220	0.24	0.77	0.28	0.20	0.10	0.30	0.43	0.33	0.42	0.58
230	0.11	0.25	0.11	0.07	0.04	0.10	0.15	0.11	0.13	0.19
240	0.06	0.07	0.05	0.03	0.02	0.04	0.07	0.05	0.05	0.06
250	0.05	0.04	0.03	0.02	0.02	0.02	0.05	0.04	0.03	0.04
280	0.03	0.03	0.02	0.10	0.01	0.01	0.04	0.03	0.02	0.03

	41	42	43	44	45	46	47	48	49	50
220	0.86	0.31	0.14	0.24	0.23	0.37	0.36	0.43	0.28	0.40
230	1.15	0.14	0.11	0.12	0.10	0.13	0.11	0.13	0.09	0.12
240	0.30	0.07	0.11	0.10	0.10	0.10	0.10	0.10	0.08	0.10
250	0.14	0.06	0.09	0.08	0.08	0.08	0.08	0.08	0.07	0.08
280	0.08	0.04	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.05

Thlaspi alpestre

a) High speed supernatant

Wavelength (nm)	Absorbance
210	0.39
220	> 2
230	> 2
240	> 2
250	> 2
280	0.50

Cd content = 0·05 µg/ml

Zn content = 128.0 µg/ml

Corrected for wet weight and volume :

Cd content = 0.1 µg/g

Zn content = 266.7 µg/g

b) Thalspi aerial parts acid digest

	Concentration µg/g	
	Cd	Zn
Dry weight	106.2	8850.2
Wet weight	15.7	1307.2

c) Thlaspi fractions collected from Sephadex G-50 preparative gel filtration column

	Absorbance									
	Fraction number									
Wavelength (nm)	1	2	3	4	5	6	7	8	9	10
220	0.31	0.35	0.15	0.21	0.22	0.21	0.27	0.21	0.23	0.25
230	0.19	0.18	0.12	0.14	0.13	0.15	0.16	0.14	0.15	0.15
240	0.12	0.10	0.08	0.08	0.08	0.10	0.10	0.08	0.09	0.09
250	0.09	0.07	0.06	0.06	0.06	0.09	0.08	0.07	0.07	0.07
280	0.06	0.05	0.04	0.04	0.04	0.06	0.05	0.04	0.04	0.04

Thlaspi fractions continued

Absorbance

Fraction number

Wavelength (nm)	11	12	13	14	15	16	17	18	19	20
220	0.17	0.21	0.33	1.93	1.54	1.75	> 2	> 2	> 2	1.05
230	0.10	0.16	0.21	0.72	0.61	0.79	1.37	> 2	1.54	0.85
240	0.08	0.10	0.12	0.25	0.26	0.36	0.55	0.70	0.58	0.37
250	0.07	0.08	0.10	0.17	0.19	0.24	0.34	0.41	0.37	0.27
280	0.05	0.05	0.06	0.12	0.12	0.15	0.21	0.26	0.25	0.21

	21	22	23	24	25	26	27	28	29	30
220	0.96	0.81	0.48	0.49	0.40	0.22	0.24	0.20	0.17	0.18
230	0.48	0.41	0.25	0.22	0.19	0.16	0.15	0.14	0.13	0.13
240	0.24	0.20	0.13	0.11	0.10	0.10	0.10	0.09	0.08	0.08
250	0.19	0.17	0.10	0.08	0.07	0.07	0.07	0.07	0.06	0.06
280	0.15	0.14	0.09	0.06	0.06	0.06	0.05	0.05	0.04	0.04

	31	32	33	34	35	36	37	38	39	40
220	0.14	0.20	0.21	0.27	0.11	0.14	0.25	0.48	0.11	0.15
230	0.13	0.12	0.13	0.13	0.14	0.11	0.11	0.13	0.19	0.18
240	0.08	0.08	0.08	0.08	0.07	0.06	0.07	0.07	0.07	0.07
250	0.06	0.06	0.05	0.05	0.05	0.05	0.04	0.05	0.04	0.05
280	0.04	0.04	0.04	0.03	0.03	0.05	0.03	0.03	0.03	0.03

	41	42	43	44	45
220	0.17	0.32	0.08	0.13	0.00
230	0.11	0.10	0.15	0.12	0.06
240	0.06	0.07	0.05	0.07	0.05
250	0.04	0.04	0.04	0.05	0.04
280	0.02	0.03	0.03	0.04	0.03

d) Thlaspi DEAE fractions

Absorbance

Fraction number

Wavelength (nm)	1	2	3	4	5	6	7	8	9	10
220	1.98	0.49	0.31	0.29	0.25	0.15	1.10	1.59	1.56	0.34
230	1.37	0.31	0.24	0.27	0.32	0.25	0.48	0.69	0.64	1.70
240	0.75	0.24	0.20	0.24	0.26	0.20	0.22	0.22	0.18	1.38
250	0.51	0.20	0.16	0.19	0.20	0.15	0.14	0.12	0.10	0.39
280	0.36	0.15	0.12	0.14	0.15	0.10	0.11	0.08	0.06	0.15

	11	12	13	14	15	16	17	18	19	20
220	1.58	0.97	0.83	0.62	0.00	0.00	0.18	0.05	0.00	0.07
230	0.65	0.33	0.28	0.24	0.08	0.06	0.04	0.07	0.05	0.09
240	0.23	0.14	0.12	0.12	0.08	0.07	0.07	0.08	0.07	0.07
250	0.15	0.10	0.08	0.09	0.06	0.06	0.06	0.06	0.05	0.05
280	0.11	0.07	0.06	0.07	0.04	0.04	0.04	0.04	0.04	0.04

	21	22	23	24	25	26	27	28	29	30
220	0.06	0.12	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.01
230	0.09	0.06	0.06	0.04	0.05	0.06	0.05	0.04	0.07	0.06
240	0.07	0.08	0.09	0.08	0.07	0.07	0.06	0.06	0.07	0.06
250	0.05	0.07	0.08	0.06	0.06	0.06	0.05	0.05	0.06	0.05
280	0.04	0.04	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.03

	31	32	33	34	35	36	37	38	39	40
220	0.03	0.00	0.06	0.06	0.06	0.00	0.09	0.00	0.00	0.29
230	0.07	0.02	0.10	0.10	0.09	0.05	0.08	0.00	0.00	0.13
240	0.06	0.06	0.08	0.08	0.08	0.06	0.06	0.05	0.05	0.07
250	0.05	0.04	0.06	0.06	0.06	0.05	0.05	0.04	0.04	0.05
280	0.03	0.03	0.04	0.04	0.04	0.03	0.03	0.03	0.03	0.03

Thlaspi DEAE fractions continued

	Absorbance			
	Fraction number			
Wavelength (nm)	41	42	43	44
220	0.00	0.00	0.00	0.00
230	0.00	0.00	0.00	0.00
240	0.04	0.03	0.03	0.03
250	0.04	0.04	0.03	0.04
280	0.03	0.02	0.02	0.02

Silene vulgaris

a) High speed supernatant

Wavelength (nm)	Absorbance
220	> 2
230	> 2
240	> 2
250	> 2
280	> 2

Cd content = 0.01 µg/ml

Pb content = 7.1 µg/ml

Zn content = 1.8 µg/ml

Corrected for wet weight and volume :

Cd content = 0.03 µg/g

Pb content = 22.1 µg/g

Zn content = 5.6 µg/g

b) Silene : Aerial parts acid digest

	Concentration µg/g		
	Cd	Pb	Zn
Wet weight	N.D.	12.3	70.0
Dry weight	N.D.	45.8	260.9

c) Silene : Fractions collected from Sephadex G-50 preparative gel filtration column

Absorbance

Fraction number

Wavelength (nm)	1	2	3	4	5	6	7	8	9	10
220	0.16	0.15	0.4	0.15	0.14	0.85	0.71	0.29	0.22	0.24
230	0.10	0.12	0.12	0.15	0.14	0.68	0.50	0.22	0.18	0.18
240	0.07	0.09	0.09	0.12	0.11	0.52	0.34	0.13	0.12	0.12
250	0.05	0.07	0.07	0.09	0.09	0.48	0.30	0.11	0.10	0.09
280	0.04	0.05	0.05	0.07	0.06	0.38	0.22	0.08	0.08	0.07

	11	12	13	14	15	16	17	18	19	20
220	0.16	0.18	0.22	0.34	0.57	1.05	1.67	1.96	1.48	1.01
230	0.16	0.18	0.21	0.29	0.44	0.76	1.11	1.16	0.88	0.64
240	0.11	0.13	0.16	0.20	0.30	0.49	0.67	0.65	0.49	0.37
250	0.10	0.10	0.12	0.15	0.22	0.35	0.45	0.46	0.37	0.29
280	0.07	0.07	0.09	0.11	0.19	0.25	0.33	0.38	0.36	0.31

	21	22	23	24	25	26	27	28	29	30
220	0.67	0.51	0.55	0.40	0.45	0.25	0.26	0.24	0.23	0.12
230	0.44	0.34	0.35	0.23	0.21	0.16	0.15	0.14	0.14	0.08
240	0.26	0.20	0.22	0.13	0.12	0.11	0.10	0.09	0.09	0.06
250	0.21	0.16	0.19	0.10	0.09	0.08	0.07	0.07	0.07	0.05
280	0.23	0.18	0.20	0.11	0.08	0.07	0.06	0.06	0.06	0.05

	31	32	33	34	35	36	37	38	39	40
220	0.04	0.04	0.04	0.05	0.05	0.07	0.10	0.07	0.35	0.04
230	0.15	0.09	0.09	0.09	0.09	0.09	0.10	0.10	0.16	0.09
240	0.11	0.07	0.07	0.07	0.07	0.08	0.07	0.08	0.08	0.07
250	0.07	0.06	0.05	0.05	0.06	0.05	0.05	0.06	0.06	0.05
280	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.04	0.04

Silene G-50 fractions continued

	Absorbance				
	Fraction number				
Wavelength (nm)	41	42	43	44	45
220	0.12	0.04	0.15	0.12	0.15
230	0.10	0.09	0.11	0.11	0.11
240	0.07	0.07	0.07	0.09	0.08
250	0.06	0.06	0.06	0.07	0.06
280	0.04	0.04	0.04	0.05	0.04

d) Silene : Fractions collected from DEAE column

	Absorbance									
	Fraction number									
Wavelength (nm)	1	2	3	4	5	6	7	8	9	10
220	1.83	1.01	0.93	1.32	1.42	0.91	> 2	> 2	> 2	1.97
230	0.99	0.49	0.51	0.80	0.76	0.54	0.73	1.37	1.10	0.73
240	0.72	0.33	0.35	0.50	0.47	0.36	0.35	0.55	0.49	0.38
250	0.58	0.28	0.29	0.38	0.36	0.29	0.27	0.38	0.36	0.30
280	0.46	0.23	0.26	0.33	0.30	0.25	0.23	0.32	0.30	0.25
	11	12	13	14	15	16	17	18	19	20
220	1.59	1.13	0.76	0.71	0.63	0.49	0.48	0.45	0.48	0.48
230	0.63	0.49	0.39	0.41	0.42	0.33	0.31	0.29	0.30	0.29
240	0.35	0.30	0.27	0.30	0.32	0.26	0.24	0.23	0.24	0.23
250	0.29	0.25	0.24	0.25	0.27	0.23	0.21	0.20	0.21	0.20
280	0.25	0.22	0.21	0.21	0.22	0.19	0.19	0.18	0.19	0.18

Silene : DEAE fractions continued

Absorbance

Fraction number

Wavelength (nm)	21	22	23	24	25	26	27	28	29	30
220	0.49	0.52	0.53	0.57	0.61	0.52	0.54	0.61	0.62	0.85
230	0.29	0.30	0.32	0.32	0.32	0.30	0.31	0.32	0.32	0.42
240	0.23	0.23	0.25	0.24	0.23	0.23	0.24	0.23	0.23	0.28
250	0.20	0.20	0.23	0.21	0.20	0.20	0.21	0.20	0.20	0.24
280	0.18	0.18	0.20	0.18	0.18	0.18	0.18	0.18	0.18	0.21

	31	32	33	34	35	36
220	0.66	0.32	0.42	0.45	0.49	0.46
230	0.43	0.29	0.31	0.31	0.31	0.30
240	0.34	0.25	0.26	0.26	0.26	0.25
250	0.27	0.23	0.22	0.23	0.23	0.22
280	0.22	0.20	0.19	0.20	0.20	0.19

Goginan. Agrostis tenuis

a) High speed supernatant

Wavelength (nm)	Absorbance
220	> 2
230	> 2
240	> 2
250	> 2
280	> 2

Cu content = 1.5 µg/ml

Cu content corrected for volume and wet weight = 3.4 µg/g

Cu content of whole plant = 16.6 µg/g wet weight and 192.3 µg/g (DW)

b) Goginan : Fractions collected from Sephadex G-50 preparative gel filtration column

Absorbance

Fraction number

Wavelength (nm)	1	2	3	4	5	6	7	8	9	10
220	1.09	0.14	0.28	0.05	0.07	0.12	0.07	0.05	0.05	0.07
230	0.45	0.24	0.26	0.20	0.20	0.24	0.20	0.19	0.19	0.20
240	0.22	0.21	0.20	0.19	0.17	0.19	0.17	0.16	0.17	0.17
250	0.17	0.18	0.18	0.17	0.15	0.17	0.15	0.15	0.15	0.16
280	0.15	0.16	0.16	0.15	0.14	0.15	0.14	0.14	0.14	0.14
	11	12	13	14	15	16	17	18	19	20
220	0.15	0.05	0.07	0.15	0.73	1.96	> 2	> 2	> 2	> 2
230	0.22	0.20	0.21	0.25	0.42	0.86	1.99	> 2	> 2	0.88
240	0.18	0.19	0.19	0.20	0.26	0.38	0.55	0.74	0.52	0.28
250	0.16	0.16	0.17	0.18	0.21	0.26	0.30	0.29	0.24	0.19
280	0.14	0.15	0.15	0.16	0.17	0.19	0.20	0.22	0.20	0.17
	21	22	23	24	25	26	27	28	29	30
220	0.81	0.34	0.23	0.68	0.07	0.03	0.08	0.01	0.06	0.04
230	0.38	0.25	0.23	0.34	0.18	0.16	0.18	0.16	0.17	0.17
240	0.20	0.18	0.17	0.19	0.15	0.15	0.15	0.14	0.15	0.15
250	0.17	0.16	0.16	0.16	0.14	0.13	0.14	0.13	0.14	0.14
280	0.15	0.15	0.14	0.14	0.13	0.12	0.12	0.12	0.13	0.13
	31	32	33	34	35	36	37	38	39	40
220	0.09	0.05	0.00	0.02	0.00	0.01	0.01	0.03	0.04	0.03
230	0.18	0.17	0.15	0.16	0.15	0.16	0.16	0.15	0.16	0.16
240	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
250	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
280	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12

Goginan : G-50 fractions continued.

	Absorbance							
	Fraction number							
Wavelength (nm)	41	42	43	44	45	46	47	48
220	0.01	0.03	0.17	0.11	0.02	0.06	0.06	0.02
230	0.16	0.16	0.19	0.18	0.16	0.17	0.17	0.16
240	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
250	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
280	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12

C) Goginan : Fractions collected from DEAE column

	Absorbance									
	Fraction number									
Wavelength (nm)	1	2	3	4	5	6	7	8	9	10
220	0.43	0.32	0.41	0.53	0.65	0.96	> 2	> 2	> 2	1.92
230	0.25	0.19	0.23	0.33	0.38	0.40	0.77	0.63	1.08	0.61
240	0.18	0.13	0.15	0.21	0.24	0.20	0.25	0.20	0.31	0.21
250	0.14	0.10	0.12	0.16	0.17	0.14	0.15	0.11	0.18	0.14
280	0.10	0.08	0.09	0.11	0.13	0.10	0.10	0.08	0.13	0.10
	11	12	13	14	15	16	17	18	19	20
220	1.36	1.09	0.92	0.55	0.62	0.48	0.37	0.35	0.36	0.32
230	0.43	0.37	0.33	0.23	0.29	0.29	0.21	0.20	0.19	0.18
240	0.17	0.17	0.15	0.13	0.18	0.21	0.15	0.15	0.13	0.13
250	0.12	0.13	0.11	0.11	0.14	0.17	0.12	0.12	0.10	0.11
280	0.09	0.10	0.09	0.08	0.11	0.13	0.08	0.09	0.07	0.08

Goginan : DEAE fractions continued

Absorbance

	Fraction number									
Wavelength (nm)	21	22	23	24	25	26	27	28	29	
220	0.39	0.29	0.32	0.30	0.43	0.43	0.54	0.48	0.38	0.42
230	0.20	0.17	0.18	0.18	0.20	0.23	0.26	0.23	0.18	0.20
240	0.13	0.12	0.12	0.13	0.13	0.15	0.17	0.14	0.12	0.12
250	0.10	0.09	0.09	0.10	0.10	0.11	0.12	0.11	0.09	0.09
280	0.07	0.06	0.07	0.08	0.07	0.07	0.08	0.08	0.06	0.07

	31	32	33	34	35
220	0.38	0.30	0.16	0.20	0.22
230	0.19	0.17	0.15	0.16	0.16
240	0.12	0.12	0.11	0.13	0.13
250	0.09	0.09	0.09	0.10	0.10
280	0.06	0.07	0.07	0.07	0.07

Molecular weight determination by Sephadex G-25 fine column

Due to a shortage of suitable molecular weight standards, only bacitracin was used. This standard was collected at fraction 8, T. alpestre at fractions 12 to 13, Goginan at fractions 12 to 14 and S. vulgaris at fractions 8 to 10.